

**RECOMBINANT HUMAN ADENOVIRUS TYPE 5
VACCINE VECTORS
EXPRESSING
RHABDOVIRAL GLYCOPROTEINS**

By

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ADENOVIRUS VECTORS EXPRESSING RHABDOVIRAL GLYCOPROTEINS

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TITLE: **Recombinant Human Adenovirus Type 5 Vaccine Vectors Expressing**
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ABSTRACT:

Rabies is an important causative agent of disease resulting in an acute infection of the nervous system and death of the individual. Vaccination of wildlife species, which are vectors of the disease, can reduce the number of non-protected animals so that the virus is not sustained in nature. Vaccines currently distributed to North American wildlife do not effectively immunize all at-risk species. This project pursued the development of recombinant human adenovirus type 5 (hAd5) expressing rabies glycoprotein as an effective vaccine vector against rabies.

Prevec *et al.* (1990) had previously described AdRG1 expressing the rabies (ERA) glycoprotein gene from the early 3 region. Low levels of rabies glycoprotein were detected in cultured cells although good titres of rabies neutralizing antibodies were measured in mice and skunks (Prevec *et al.*, 1990; Charlton *et al.*, 1992). Using AdRG1 as a guide, I examined conditions required for optimal rabies glycoprotein expression. Vectors AdSVRG1.3, AdRG1.2, AdRG4, AdRG1.3, and AdHCMV13RG, containing variable exogenous promoters and genomic deletions, were constructed and evaluated. Although no formula was derived to predict optimal vector structure, AdRG1.3, containing rabies glycoprotein and SV40 polyadenylation sequences in parallel orientation to the E3 promoter, demonstrated high levels of glycoprotein expression and elicited high levels of neutralizing antibodies.

Rabies glycoprotein shares structural and functional properties with the glycoprotein of vesicular stomatitis virus, also a rhabdovirus. We predicted that chimaeric glycoprotein molecules would structurally resemble the parental glycoproteins and, thus, be useful as vaccines. Five chimaeric DNA sequences of the two glycoproteins were designed and expressed from hAd5 vectors. The chimaeric proteins were expressed at low levels in infected cultured cells yet no anti-rabies nor anti-VSV neutralizing antibodies were detected in serum from immunized BALB/c mice. A block in the post-translational processing pathway of the chimaeric proteins at the level of oligosaccharide modifications may also be a result of blocked movement of proteins.

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ABBREVIATIONS

A	adenine
aa	amino acids
Ad	adenovirus
ADCC	antibody-dependent cell-mediated cytotoxicity
Ala	alanine
Amp ^R	ampicillin resistance
araC	cytosine β -D-arabinofuranoside
Arg	arginine
Asn	asparagine
Asp	asparagine
CIAP	calf intestinal alkaline phosphatase
CNBr	cyanogen bromide
CPE	cytopathic effect
CTL	cytotoxic T lymphocytes
CVS	challenge virus strain
Cys	cysteine
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dNTP	dATP + dCTP + dGTP + dTTP
dTTP	deoxythymidine triphosphate
E1	early region 1
E3	early region 3
ER	endoplasmic reticulum
ERA	Evelyn-Rokitnicki-Abelseth
E.U.	equivalent units; defined on page 85
G	rabies or VSV glycoprotein
Gln	glutamine
Glu	glutamic acid
Gly	glycine
Gs	soluble glycoprotein
hAd2	human adenovirus type 2
hAd5	human adenovirus type 5
IFN γ	gamma interferon
IL-2	interleukin-2
Ile	isoleucine
IM	intramuscular
Ind	Indiana serotype
IP	intraperitoneal

IU	international units; defined on page 85
kb	kilobase
kD	kilodaltons
L	large protein
Leu	leucine
Lys	lysine
M	matrix protein
MAb	monoclonal antibody
Met	methionine
MLP	major late promoter
MHC	major histocompatibility complex
mu	map units
N	nucleocapsid
NJ	New Jersey serotype
Nm ^R	neomycin resistance
nt	nucleotide(s)
P	phosphoprotein
pfu	plaque forming units
p.i.	post-infection
Phe	phenylalanine
pro	promoter
Pro	proline
RT	room temperature
SAD	Street-Alabama-Dufferin
SC	subcutaneous
Ser	serine
T	thymine
Thr	threonine
ts	temperature sensitive
Val	valine
VNA	virus neutralizing antibody
VSV	vesicular stomatitis virus
VV	vaccinia virus
v/v	volume per volume
w/v	weight per volume
X	nonspecified amino acid

CHAPTER 1

INTRODUCTION

HUMAN ADENOVIRUS TYPE 5 AS A VACCINE VECTOR AGAINST RHABDOVIRUSES

The rhabdoviruses, rabies and vesicular stomatitis virus, are both important as causative agents of disease in animals and man. Rabies virus causes an acute infection of the mammalian nervous system leading to death. The virus is maintained in wild animal species and interferes with human activities through infection of domestic animals or of humans directly. Geographical areas in which rabies is endemic must maintain expensive surveillance and control programs to reduce the probability of infection as well as effective treatment programs if contact has occurred. It is estimated that over 50,000 individuals die annually of rabies infection (Wilde *et al.*, 1994), primarily in SE Asia and other tropical countries (Warrell and Warrell, 1988). In Ontario, where no deaths from rabies have occurred for many years, millions of dollars are spent annually on rabies prevention. Vesicular stomatitis virus, while not a natural pathogen of man, is an economically important agricultural problem in some parts of the world, particularly in Central America.

Vaccination continues to be the only effective way to prevent or to treat rhabdovirus infection. The objective of vaccinating wildlife species against rabies is to reduce the number of non-protected animals to the point where the disease cannot sustain itself in nature. Rabies virus vaccines, consisting of killed or highly attenuated rabies virus, have been available since the time of Pasteur. Attenuated rabies strains currently used to vaccinate North American wildlife fail to effectively immunize several vector species and are pathogenic in rodents (Wandeler *et al.*, 1988; Rupprecht *et al.*, 1990; Artois *et al.*, 1992; and others). To overcome some of these problems rabies vaccine research has emphasized the development of recombinant viruses as vaccine vectors to express rabies glycoprotein. Human adenovirus type 5 (hAd5), as a viral vector expressing foreign antigens, has many desirable properties for use as a recombinant vaccine

(reviewed by Graham and Prevec, 1991, 1992). This project concentrates on the development of hAd5 as a viral vector expressing the rabies glycoprotein. Due to similarities in glycoprotein structure between rabies and vesicular stomatitis virus (VSV), the prototype rhabdovirus, recombinant hAd5 expressing chimaeric rabies/VSV glycoproteins were also generated. hAd5 vectors expressing rhabdoviral sequences were constructed and evaluated as potential vaccines to protect against rabies infections. This review serves to introduce the reader to the rhabdoviruses rabies and vesicular stomatitis virus, as well as to adenoviruses as vaccine vectors.

RABIES DISEASE

The transfer of rabies virus between animals occurs as a result of transfer of infected saliva by the bites of rabid animals. The virus may be locally sequestered in striated muscle tissue for days or months where replication can occur (reviewed by Murphy, 1985; Shope and Tesh, 1987; Wunner, 1987; Tsiang, 1993). In humans the period between introduction of virus and appearance of disease can last from 15 days to a year. To initiate the disease state, rabies must be transmitted to the central nervous system either through exposed sensory nerve endings or through motor end plates (myoneural junctional organs). The precise neuronal receptor for rabies has not been clearly determined though evidence supports the acetylcholine receptor or a rhabdovirus common receptor (Wunner *et al.*, 1984a; Murphy, 1985; Koprowski, 1988). Levels of acetylcholine receptors may reflect differences in cell susceptibility to infection (Baer *et al.*, 1990). In the absence of an animal bite, rabies can be transmitted by abrasion to target sensory nerve endings of epithelial and subepithelial tissues, or by aerosol (for example, in bat-infested caves) to infect olfactory neuroepithelial cells (Constantine *et al.*, 1972). Cases of rabies transmission by infected corneal grafts have also been reported (Warrell and Warrell, 1988). After entry into the central nervous system the virus undergoes centripetal passage through axons to the brain. Transmission between neuronal junctions occurs by release of virus into tissue spaces and subsequent reentry by pinocytosis, or by membrane fusion and direct passage of the genome moiety. The virus also travels from peripheral nerves to replicate in mucogenic organs, such as the salivary gland, resulting in high titers of virus release into glandular ducts and into the saliva (Campbell and Zhang, 1993; Tsiang, 1993).

In humans, infection can result in either a furious rabies disease or paralytic /dumb/ rabies: both are acute infections resulting in death. Furious rabies is characterized by three clinical phases. The initial phase consists of nonspecific symptom such as chills, fever, photophobia, wasting syndrome, anorexia, and abnormal sensations around the wound site. A second phase involves neurological perturbances such as behavior disturbance, hyperventilation, hydrophobia, and hallucinations, and is followed by a final comatose phase and death (reviewed by Wunner, 1987).

RABIES VECTORS

The major reservoirs of rabies in North America and Europe are wild carnivores such as the wolf, fox, coyote, jackal, and bobcat, although the virus is problematic in domestic animals, cattle, and bat populations (Shope and Tesh, 1987; Wiktor *et al.*, 1988; Campbell and Zhang, 1993). In a defined geographical area, rabies is found predominantly in a single vector species (Blancou *et al.*, 1986; Campbell and Zhang, 1993). In North America there are four geographically dominant vectors: raccoons (*Procyon lotor*) in the eastern/southeastern USA, skunks (*Mephitis mephitis*) in central USA and in Canada, and arctic foxes (*Alopex lagopus*) and red foxes (*Vulpes vulpes*) in the arctic and eastern Canada, respectively (Campbell and Zhang, 1993). Skunks are important vectors of rabies virus in eastern Canadian cities outnumbering the fox population (Rosatte *et al.*, 1987). In Ontario the enzootic zone for rabies virus comprises an area of approximately 98,000 km² of primarily agricultural land (MacInnes *et al.*, 1988). Direct encounters with wild animals, or (more probably) with exposed domestic animals such as dogs and cats results in disease transmittance to humans. The vaccination of dogs and cats against rabies is considered a major medical and public health expense to reduce the risk of human infection.

IMMUNE RESPONSE TO RABIES

Immune Response To Rabies Infection

During a natural infection with rabies, the host immune response to the infection is delayed until the later stages of the disease when a response of minimal intensity can usually be detected (reviewed by Murphy, 1985; Wunner, 1987). At the bite site very little viral antigen is present to trigger a response; further, there is minimal cytopathology in muscle and in peripheral nerves. The central nervous system provides physical barriers (the presence of a myelin sheath and the absence of lymphatics and lymphocyte trafficking) which further hinder the immune response. Both innate and adaptive immune responses can be demonstrated. Lesions in the spinal cord contain macrophages, lymphocytes, and polymorphonuclear cells. Macrophages play a role in limiting virus spread, and may promote antigen presentation. A T cell-dependent B cell response is not measured until after the development of clinical symptoms (Wunner, 1987) with serum antibody titers highest at time of death (Kasempimolporn *et al.*, 1991); this allows a rapid (within 24 hours) post-mortem diagnosis of rabies by immunofluorescence. Studies with infant cats have shown that the immune organs undergo distinct changes during rabies infection. The thymus is diminished in size but contains a disproportionately large medulla; the periarteriolar lymphatic sheath (PALS) and follicle mantles in the spleen are depleted and disorganized, and a lack of germinal centers and overall degeneration in the lymph nodes is detected (Torres-Anjel *et al.*, 1988).

Immune Response To Vaccination

In pre-exposure vaccinations serum antibody titers correlate with protection from infection (Wunner, 1987). The antibody serves three functions (reviewed by Wunner, 1987): in addition to neutralizing rabies virus (Wiktor *et al.*, 1968), antibody triggers the complement system (Wiktor and Clark, 1972) and promotes antibody-dependent cell-mediated cytotoxicity (ADCC) (Miller and Nathanson, 1977). Volunteers immunized with the rabies human diploid cell vaccine developed VNA and retained variable serum antibody levels over at least a sixty month period (Oelofsen *et al.*, 1991). The critical immune response is the development of cell-mediated

a sixty month period (Oelofsen *et al.*, 1991). The critical immune response is the development of cell-mediated immunity (reviewed by Wunner, 1987) which correlates with viral protection (Wiktor *et al.*, 1977; Wiktor, 1978). Rabies virus protein-specific CD8⁺ cytotoxic T lymphocytes can be detected 4 days post-immunization with maximal activity on day 7 (Celis *et al.*, 1985). Studies also showed that CD4⁺ cytotoxic T cell lines specific for the viral proteins could be isolated from individuals immunized with the rabies PM vaccine (Celis *et al.*, 1988). Interleukin-2 (IL-2) production after immunization was shown to correlate with rabies protective activity (Joffret *et al.*, 1991) and failed to correlate with VNA levels (Perrin *et al.*, 1991) indicating IL-2 involvement in CTL proliferation. Vaccination also induced the production of interferon-gamma, a cytokine important in post-exposure protection (Wiktor *et al.*, 1972, 1976; Baer, 1988a).

CONTROL OF RABIES

Control Of Rabies In Humans

The control and prevention of rabies disease in human and animal populations is achieved through pre-exposure vaccination programs. The inactivated rabies vaccines initially developed for use in humans were prepared in infected rabbit, sheep, or goat brains as these tissues gave high and consistent yields of virus. These vaccines, although inexpensive to produce, had several drawbacks including neurological complications, low immunogenic content, and a transmittance risk of unconventional viruses such as Creutzfeldt-Jakob disease agent (Campbell and Zhang, 1993). Present day rabies vaccines are grown in diploid, non-nervous cell culture (for example, in human MRC-5 fibroblasts), the virus is inactivated with β -propiolactone or phenol, and administered pre-exposure to individuals at high risk of infection, or given along with rabies immune globulin in a post-exposure scenario to induce a humoral response (Roumiantzeff *et al.*, 1985; Wiktor *et al.*, 1988). Pre-exposure vaccinations are administered on day 0, day 28, and a final dose one year later (Nicholson, 1990). In post-potential exposures 5-6 doses of vaccine are required; for example, the vaccine can be administered intramuscularly in the deltoid muscle at 0, 3, 7, 14, and 28 days to produce an efficient, high titer primary antibody response which

protects against disease development (Campbell and Zhang, 1993).

Control Of Rabies In Domestic Animals And Livestock

Control of rabies in domestic animals and livestock has combined vaccination programs with dog control measures (Nicholson, 1990). Initial veterinary vaccines were either of a modified-live or inactivated nervous cell origin. The modified-live vaccines were derived from the Flury strain of rabies adapted to grow in embryonated chicken eggs; both derivatives, the LEP (low egg passage) and HEP (high egg passage) strains, demonstrated pathogenicity (Wandeler *et al.*, 1988). At the present time domestic animals and cattle are vaccinated with an inactivated cell culture-derived vaccine (Campbell and Zhang, 1993). Inactivated vaccines are produced using the Street-Alabama-Dufferin (SAD; adapted to grow in hamster kidney cell culture) or derivative Evelyn-Rokitnicki-Abelseth (ERA) or Vnukovo-32 strains of rabies. A single dose of inactivated veterinary vaccine is given intramuscularly (IM) at 3 months of age and is followed by a second dose a year later.

Control Of Rabies In Wildlife

Control of rabies in wild animals has combined efforts at vaccinating at-risk species with methods aimed at reducing the wild fox population. Poisoning or killing of foxes can only serve as a temporary control measure unless sufficiently large numbers of animals are eliminated. Initial programs aimed at vaccinating wild animals were implemented in 1962 (Nicholson, 1990), and present-day efforts employ the packaging of vaccine into baiting systems such as chicken heads or small, sealed containers prior to ground- or aerial-based distribution. Baer and coworkers (1971) administered attenuated ERA rabies strain orally to foxes and observed high titers of VNA and the induction of protective immunity. Attenuated SAD strain, when given as a chicken head bait, was effective in vaccinating foxes in field studies in Switzerland (Steck *et al.*, 1982) and successfully eliminated rabies virus from that country (Wandeler *et al.*, 1988). Aerial bait drops of live attenuated SAD-ERA vaccine are carried out in Ontario using a wax and tallow coated sponge as bait (Black and Lawson, 1980; Lawson *et al.*, 1982; Johnston *et al.*,

1988). By incorporating a tetracycline marker into the bait, bait acceptance rate and actual date of ingestion could be determined by counting daily incremental lines in fox teeth dentin between the tetracycline deposit and the growing edge. A 74% rate of bait acceptance was seen with foxes while raccoons were at the low end of acceptance (2-30%) (Johnston *et al.*, 1988). A drawback of the SAD strain is its residual pathogenicity for some rodent species (Wandeler *et al.*, 1988; Artois *et al.*, 1992); this observation was previously described for the CVS strain (Correa-Giron *et al.*, 1970; Baer, 1988b). Attenuated vaccines were demonstrated to be pathogenic for skunks (Rupprecht *et al.*, 1990), and were ineffective in studies carried out with raccoons (Rupprecht *et al.*, 1986).

Drawbacks

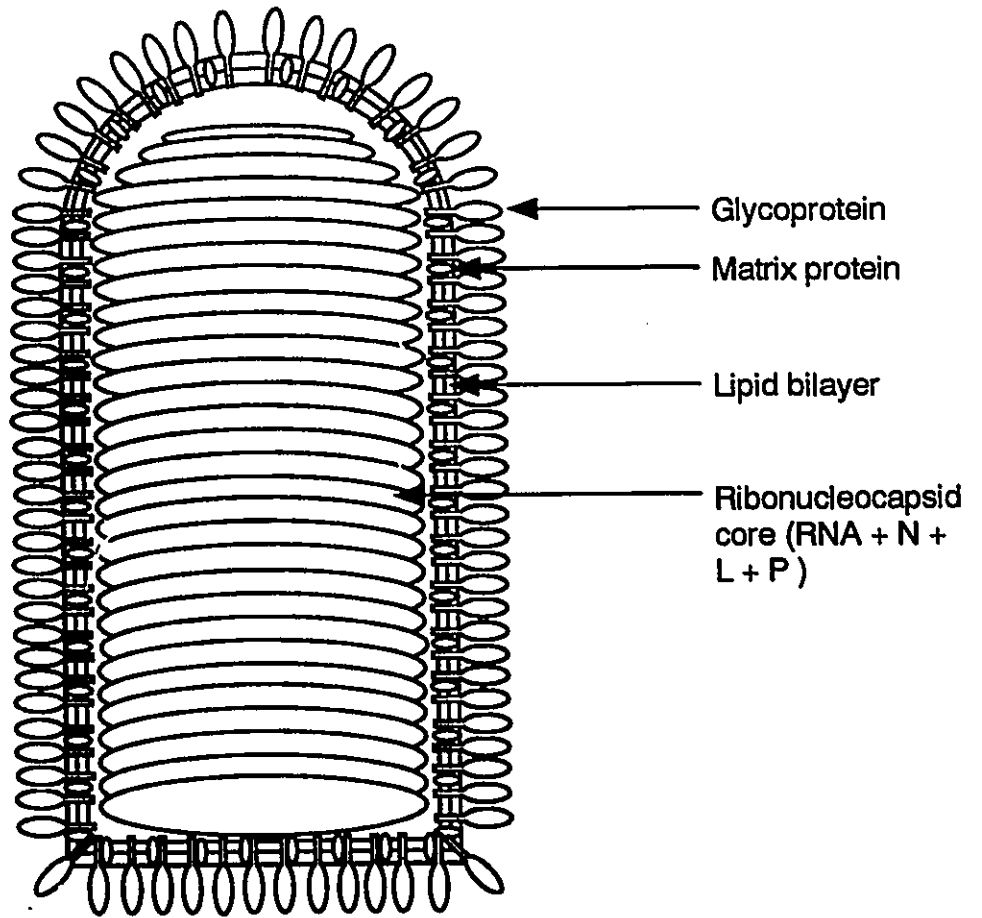
Although the vaccines for humans and for domestic animals have proven highly effective, neither has had an effect on the ecological cycle of rabies. The preparation of inactivated vaccines from virulent material poses concerns for the complete inactivation of the virus. Disadvantages of cell culture-based vaccines have included the high manufacturing and immunization costs in a large-scale eradication program, and problems ensuring thorough worldwide accessibility to the product (Campbell and Zhang, 1993). In third world countries nervous tissue-prepared vaccines are administered: the poor antigenicity requires a course of immunization of up to 24 x 5 ml injections of 5% or 10% brain suspension and results in high rates of side effects (Nicholson, 1990). New approaches to rabies vaccine development have involved the preparation of individual viral components, such as the glycoprotein, that are directly responsible for immune response and protection.

MOLECULAR COMPOSITION OF RHABDOVIRUSES

Rabies, genus *Lyssavirus*, and vesicular stomatitis virus (VSV), genus *Vesiculovirus*, are both rhabdoviruses and, as a result, have many properties in common. Rhabdoviruses are negative-strand RNA viruses with a bullet-type morphology and an average virion size of 180 nm by 75-80 nm (reviewed by Rose and Schubert, 1987; Wunner *et al.*, 1988; Tsiang, 1993). The

FIGURE 1.1: STRUCTURE OF RHABDOVIRUSES

A diagrammatic representation of a prototype rhabdovirus. The bullet-shaped virion is approximately 180 nm by 75-80 nm. The ribonucleocapsid core contains nucleoproteins (N), phosphoproteins (P), and large (L) proteins in association with a negative-strand RNA molecule. The matrix protein is found within the lipid bilayer while the glycoprotein spikes span the lipid bilayer at 4-5 nm intervals.



single-stranded noninfectious genome of 11-12 kb is complementary to messenger RNA. The virus consists of five proteins (Figure 1.1) (Mudd and Summers, 1970): the nucleoprotein (N, 50 kD), phosphoprotein (P, 30 kD), and large (L, 190 kD) proteins are part of the nucleocapsid core, the matrix protein (M, 20-30 kD) lies within the lipid membrane envelope, and the glycoprotein spikes (G, 70 kD) span the envelope. The nucleocapsid structure of N protein and RNA form a helical cylinder of 30-35 coils that associate with P protein. The L and P proteins complex into a polymerase responsible for the transcription of five messenger RNA from the ribonucleoprotein template (Banerjee and Barik, 1992). During virion assembly, the M protein interacts with and stabilizes G subunits through the cytoplasmic domain (Lyles *et al.*, 1992). The glycoprotein has several roles in the virus life cycle (Wagner, 1987): (1) it binds the host cell surface receptor and, after virus entry into the cytoplasm in vesicles, (2) the glycoprotein initiates virion uptake by low pH fusion with endosomal membranes resulting in the release of virion contents into the cytoplasm, (3) during virion assembly its cytoplasmic domain interacts with nucleocapsid core components to direct budding of the virus particle, and (4) G is the target of neutralizing antibodies. These points will be discussed in detail in later sections.

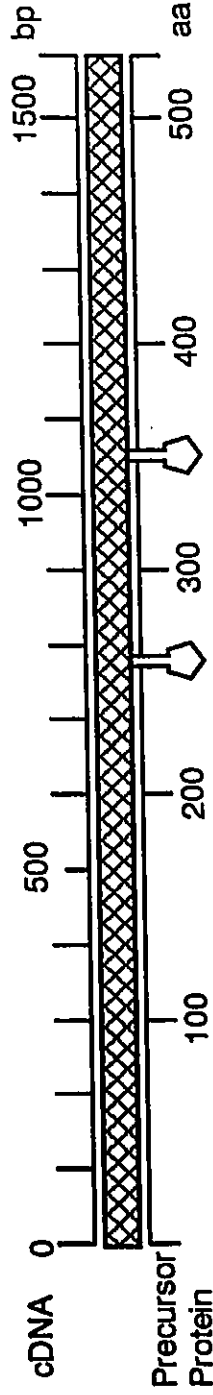
RABIES GLYCOPROTEIN

Characteristics

The glycoprotein used in this work is of the rabies ERA strain: the 1575 nucleotide sequence (Anilionis *et al.*, 1981) is translated into a precursor protein of 524 residues of which the first 19 amino acids (aa) comprise the signal sequence (reviewed in Wunner *et al.*, 1988). The mature glycoprotein (M_w 67-68 kD) contains a 22 residue carboxy-terminus membrane domain (Fig. 1.2). Rabies ERA and CVS (Challenge virus) strains share a 94.3% homology of glycoprotein ectodomain sequences with an overall homology of 90.9%. The CVS strain of rabies carries a palmitic group acylated to a transmembrane-located cysteine residue (Gaudin *et al.*, 1991a). Gaudin *et al.* (1992) confirmed a trimeric structure for native ERA rabies G and suggested a "head" and "stalk" model for the glycoprotein structure - this structural organization was confirmed for the CVS strain as well (Whitt *et al.*, 1991). Dietzschold *et al.* (1983)

FIGURE 1.2: RABIES ERA GLYCOPROTEIN

Schematic representation of rabies (ERA) glycoprotein and its immunogenic determinants. The 1575 nucleotide sequence is translated into a mature protein of 524 residues after the removal of a 19 amino acid signal peptide. Glycosylation sites at asparagine residues 266 and 338 of the precursor protein sequence are represented by inverted tree structure. Approximate locations of functional domains are indicated with solid rectangles. Residues 208 to 233 are similar to sequences found in snake neurotoxins and may be involved in rabies neurotropism. Residues 53 to 62, 217 to 219, and 352 to 376 are critical in the induction of neutralizing antibodies, while the determinant responsible for stimulating CTL maps between residues 149 and 197. Rabies glycoprotein treated with cyanogen bromide (CNBr) yields eight major fragments (indicated by double lines): Cr1, aa 20-63, Cr7, aa 76-121, Cr4, aa 122-197, Cr6, aa 207-255, Cr2, aa 263-310, Cr3, aa 311-342, Cr5, aa 343-395, and Cr2A, aa 405-471. ++ indicates properties of CNBr fragments under reduced conditions. + indicates properties of CNBr fragments under non-reduced conditions. All referrals to residue positions are based on the precursor protein sequence.



Signal peptide
 Homology to neurotoxins
 CTL determinant
 Neutralizing Ab
 IL-2 production



Cyanogen bromide fragments	Cr1	Cr7	Cr4	Cr6	Cr2	Cr3	Cr5	Cr2A
CTL determinant	-	-	+	-	-	-	-	-
Neutralizing Ab	++	-	++	-	-	++	-	-
IL-2 production	++	+	+	-	++	++	-	++

identified a subspecies of glycoprotein secreted from rabies virus-infected cells; the soluble glycoprotein, G_s (M_w 61 kD), lacked 58 aa from the carboxy terminus of the protein yet all epitopes on the wild type glycoprotein were detected. Rabies ERA G contains three potential N-glycosylation sites, although only two are used (Asn residues 266 and 338 based on the precursor protein sequence) (Fig. 1.2). At least one of the added oligosaccharides is complex containing sialic acid (Dietzschold, 1977). Rabies G expression studies in Chinese hamster ovary cells wild type or defective at various points of the N-linked oligosaccharide processing pathway (Lec2, Lec1, and Lec8) demonstrated that some form of N-glycosylation was required for transport to the cell surface although the specific structure as determined by the addition of sialic acid or high mannose groups was not critical (Burger *et al.*, 1991). Glycoprotein lacking both N-linked glycosylations was not transported to the surface.

Glycoprotein Binding Of The Cell Surface Receptor

The rabies glycoprotein is responsible for the binding of virus to the host cell. There are at least two groups of receptors on the cell surface, one of which is the acetylcholine receptor (Lentz *et al.*, 1982, 1984), and *in vitro* studies have shown that sialic acid or phospholipid groups on gangliosides may function as a second receptor group (Superti *et al.*, 1984a, 1984b, 1986). Lentz and coworkers (1982, 1984) provided *in vivo* evidence of rabies virus binding the acetylcholine receptor on nerve cells via the glycoprotein. Residues¹ 208 to 233 of the rabies glycoprotein are similar to residues 30-56 of snake venom curaremimetic neurotoxin and other neurotoxins; these regions of the neurotoxin form a loop structure important in neurotoxicity (Lentz *et al.*, 1984). The correlation was viewed as an indicator of rabies neurotropism and suggested that the acetylcholine receptor was the rabies virus receptor. Rabies glycoprotein, synthetic peptides of snake venom, and curaremimetic neurotoxins have a high affinity for residues 179-192 of the $\alpha 1$ -subunit of the nicotinic acetylcholine receptor (Lentz, 1990). Recent work by Lentz (1991) demonstrates peptides representing residues 192-222 of CVS rabies glycoprotein (and the corresponding ERA G region) and conserved loop 2 (residues 25-44) of

¹ Throughout this manuscript all referrals to residue positions within the glycoprotein are based on the precursor protein sequence.

king cobra toxin had high affinities for the *Torpedo* electric organ membrane acetylcholine receptor as determined in binding competition studies using ^{125}I - α bungarotoxin. Wunner and coworkers (1984a) identified a saturable receptor on cells of both neural and non-neural origin that enabled the binding of 3×10^3 - 15×10^3 virions per cell. Attachment was not affected by proteolytic enzymes but treatment with neuraminidase, specific for phospholipids or glycolipids, inhibited binding (Superti *et al.*, 1984a, 1984b). Competitive binding to the receptor was observed with a nonpathogenic rabies variant virus RV194-2 as with VSV, suggesting that this receptor was common to rhabdoviruses (Wunner *et al.*, 1984a). Highly sialylated gangliosides (glycosphingolipids) were also shown to be part of the membrane receptor structure for rabies by Superti *et al.* (1986); gangliosides are present on membranes of eukaryotic cells and are very abundant on nervous tissue.

Role Of Glycoprotein In Fusion

After virion endocytosis, the glycoprotein induces virion envelope fusion with endosomal membranes resulting in release of virion contents into the cytoplasm (Superti *et al.*, 1984a; reviewed in Wunner, 1987). Rabies glycoprotein is responsible for cell fusion and erythrocyte hemolysis under acidic conditions (Mifune *et al.*, 1982). The pH dependence of this property was proposed to depend on modified protein structures. It was demonstrated that G could adopt at least three configurations, each configuration conferring unique properties to the glycoprotein (Gaudin *et al.*, 1991b, 1993). Membrane fusion and hemagglutination are not detected at neutral pH at which G is not affected by bromelain or trypsin cleavage reactions. At pH 6.4, the glycoprotein becomes sensitive to proteases, virus aggregation is observed, hemagglutination levels reach a maximum, yet no fusion is detected. Cell fusion is obtained below pH 6.1. Gaudin and coworkers (1991b) report the isolation of neutralizing monoclonal antibodies (MAbs) that are able to differentiate the three forms of the glycoprotein. Whitt and coworkers (1991) have shown that rabies (CVS strain) glycoprotein expressed from cDNA in HeLa cells induced syncytia formation at acidic pH, although expression of a chimeric glycoprotein containing rabies G extracellular domain and the VSV G transmembrane and cytoplasmic domains could promote

very little detectable membrane fusion. They concluded that the chimeric protein had undergone a conformational event that interfered with the fusion activity.

Role of Glycoprotein In Virulence

Avirulent rabies CVS and ERA variants have been isolated which contain an amino acid substitution at residue 352 of the glycoprotein sequence (Seiff *et al.*, 1984; Flamand *et al.*, 1985; Tuffereau *et al.*, 1989). The substitution in the strains studied was shown as a change from arginine (Arg) to isoleucine (Ile) or glutamine (Gln), although the reintroduction of a lysine (Lys) residue restored virulence (Tuffereau *et al.*, 1989). Pathogenic and apathogenic viruses differ in the ability to infect neuroblastoma cells *in vitro* and neurons *in vivo*. Apathogenic strains demonstrate slower rates of infection and cell-to-cell spread, less cellular necrosis, and overall fewer infected neurons (Dietzschold *et al.*, 1985). Benmansour and researchers (1991) propose a potential role of Arg 352 in neuronal receptor recognition although this has yet to be shown. Arg 352 is located in neutralizing site III (Fig. 1.2) (Flamand *et al.*, 1985; described further below). Other sites are also involved in pathogenesis. Préhaud and coworkers (1989a) have characterized a tsG1 mutant of rabies CVS containing a substitution of Leu to Phe at position 151: the mutant had reduced pathogenicity in intracerebral inoculations and was avirulent in intramuscular (IM) inoculations, while in cell culture cytopathic effect (CPE) was abolished and a persistent infection was established.

RABIES GLYCOPROTEIN AND THE IMMUNE RESPONSE

Rabies glycoprotein is the main viral component recognized by the immune system. Immunization with rabies glycoprotein can induce synthesis of neutralizing antibody (Wiktor *et al.*, 1973a; Wunner *et al.*, 1983) and protection against a lethal infection with rabies virus. Further, rabies glycoprotein is also the target protein for rabies virus-specific cytotoxic T lymphocytes (CTL) (Macfarlan *et al.*, 1984; Wiktor *et al.*, 1984), and has been shown to stimulate the production of cytokines interferon (Baer *et al.*, 1979) and interleukin-2 (IL-2) by helper T cells (Macfarlan *et al.*, 1984; Wunner *et al.*, 1985). The soluble glycoprotein, G_s, fails

to protect mice against a lethal challenge (Dietzschold *et al.*, 1983). Unlike wild type G, G₁ is unable to aggregate in the presence of *n*-octylglucoside, a property that is important for immunogenic potential (Wunner *et al.*, 1983).

Induction Of Neutralizing Antibody

Initial characterizations of antigenic sites on rabies glycoprotein responsible for induction of virus neutralizing antibodies were done by analyzing cyanogen bromide (CNBr) cleavage fragments of the glycoprotein (Dietzschold *et al.*, 1982). Treatment of glycoprotein with CNBr under reducing conditions yields eight major fragments (Fig. 1.2): Cr1, aa 20-63; Cr7, aa 76-121; Cr4, aa 122-197; Cr6, aa 207-255; Cr2, aa 263-310; Cr3, aa 311-342; Cr5, aa 343-395; and Cr2A, aa 405-471. Both Cr1 and Cr3 were immunoprecipitated with polyserum suggesting that these fragments were similar in their native and reduced conformations. Significant but low titers of virus neutralizing activity (VNA) were observed with fragments Cr1, Cr4, and Cr3 (180, 510, and 270 VNA units, respectively) when compared with the native glycoprotein (9,000 VNA units). Two disulphide bonds were predicted to contribute to the native conformation: purification of non-reduced G fragments resulted in fractions Ca1, Ca2, and Ca3, which, upon reduction, contained fragments Cr1+Cr6, Cr2+Cr6+Cr1, and Cr2+Cr6, respectively (Dietzschold *et al.*, 1982). Dietzschold and coworkers (Dietzschold *et al.*, 1982; Wunner *et al.*, 1983) concluded that Cr6 was linked by disulphide bonds to both Cr1 and Cr2, placing Cr4 within a loop structure.

A second approach to locate neutralizing epitopes of the glycoprotein was to identify point mutations in neutralization-resistant variant viruses. Neutralization-resistant variant viruses were grouped on the basis of binding studies with panels of monoclonal antibodies (MAbs) directed against rabies: rabies ERA and CVS glycoproteins share four antigenic sites (I, II, III, and VI) while sites IV and V are ERA type-specific (Lafon *et al.*, 1983; Wunner *et al.*, 1984b). Site I was topologically distinct, while sites II (comprising IIA, IIB, and IIC) and III (IIIA, IIIB) were in close proximity (Lafon *et al.*, 1983). Sites I, II, and III are responsible for the majority of anti-rabies neutralizing antibodies (Lafon *et al.*, 1983, 1984). Site III is considered the major neutralizing site having the largest representation of virus-neutralizing epitopes (Lafon *et al.*,

1983, 1984). In a study of rabies glycoprotein antigenicity in H-2^d mice, Benmansour *et al.* (1991) found that 97% of 264 isolated MAbs recognized sites II or III. The stimulation of neutralizing antibodies is G conformation dependent, and individual sites were not recognized in decreased pH conditions (Gaudin *et al.*, 1991b), although Bunschoten *et al.* (1989a), using MAb 6-15C4, have described a non-conformation dependent epitope common to both CVS and ERA strains of rabies.

Préhaud *et al.* (1988) isolated rabies CVS derivative strains expressing mutant G that were not neutralized by a panel of 12 MAbs directed against antigenic site II. Sequencing studies determined that the neutralization-resistant viruses contained one of seven identified amino acid substitutions between residues 53 and 62 and three amino acid substitutions between residues 217 through 219. Mutations were also observed at amino acids 166 and 203 clearly indicating that site II was a discontinuous structure (Fig. 1.2). Site III is predominantly continuous in nature as defined by nonneutralizing point mutations at positions 352, 355, 357, and 376; the epitope defined by residue 376 may be related to the rest of site III by a foldback event (Lafon *et al.*, 1983).

T_HHELPER Epitopes

CNBr fragments of rabies glycoprotein were evaluated for potential effects on T cells. Macfarlan and coworkers (Macfarlan *et al.*, 1984; Wunner *et al.*, 1985) showed that three of these denatured fragments (Cr1, residues 20-63; Cr2 + Cr2A, residues 263-310 + 405-471; Cr3, residues 311-342) stimulated antigen-specific proliferation of virus-primed helper T lymphocytes and the production of IL-2. Using synthetic peptides R20 and R21 the region of Cr1 responsible for proliferation was further narrowed down to residues 51-63; these peptides were also found to stimulate a subpopulation of Lyt-2⁺ cells. Synthetic peptides of Cr2 or Cr3 failed to induce proliferation and this was explained as due to a lack of N-glycosylation of the glycoprotein. Non-reduced fragments Ca4 (corresponding to Cr4) and Ca6 (corresponding to Cr7) also induced T cell proliferation (Wunner *et al.*, 1985). Rabies virus-specific CD4⁺ T cell clones isolated from the PMBC of a vaccinated human donor were demonstrated to provide help for an *in vitro*

antibody response against neutralizing determinants on the glycoprotein (Bunschoten *et al.*, 1989b).

Cytotoxic T Lymphocyte Epitopes

In addition to neutralizing antibody, a cell-mediated response directed against live or chemically inactivated virus can protect against rabies infection (Wiktor *et al.*, 1977; Wiktor, 1978). By expressing rabies ERA glycoprotein from a vaccinia vector, Wiktor and coworkers (1984) demonstrated that a significant portion of rabies-induced CTL was directed against the glycoprotein. Vaccinia vectors expressing defective glycoprotein failed to induce a CTL response (Wiktor *et al.*, 1984). The Ca4 peptide (corresponding to reduced fragment Cr4) stimulates the proliferation of a proportion of lymphocytes expressing Lyt 2 antigen that is associated with cytotoxic function (Macfarlan *et al.*, 1984; Wunner *et al.*, 1985). Macfarlan *et al.* (1986) presented ERA G in lipid vesicles to A/J mice and evaluated the CTL response: glycoprotein was required in higher doses to initiate a CTL response. Cleavage of G with cyanogen bromide or limited tryptic digestion enhanced the CTL response while reduction and alkylation of the glycoprotein destroyed CTL activity. The glycoprotein determinant responsible for stimulating CTL was localized between residues 149 and 197, a region incorporating a single disulphide loop. Induction of CTL by G in a liposome preparation was carried out by Kawano *et al.* (1990): CTL clones were H-2K^k restricted and produced low levels of gamma IFN in response to antigen stimulation. Transfer of G-specific CTL to syngeneic mice infected with ERA protected approximately 50% of the mice over a four week period (Kawano *et al.*, 1990).

VACCINES BASED ON RABIES GLYCOPROTEIN

The use of glycoprotein in vaccine preparations independent of other rabies components provides a means of ensuring a lack of virulence and associated complications. Effective presentation of the glycoprotein in a three-dimensional conformation ensures efficient recognition of discontinuous neutralizing epitopes. Expression systems have been developed that allow large-scale purification of the glycoprotein in *Escherichia coli* (Yelverton *et al.*, 1983; Lathe *et al.*,

1984; Malek *et al.*, 1984), yeast (Lecocq *et al.*, 1984; Klepfer *et al.*, 1993), and baculovirus-infected insect cells (Préhaud *et al.*, 1989b). The administration of purified glycoprotein as a vaccine resulted in low levels of immunity due to incorrect G conformation than that found in the virion state and problems of achieving site-directed administration (Brown, 1984; Fields and Chanock, 1989). In addition, expression of rabies G in bacterial systems failed to obtain post-translationally processed protein (Yelverton *et al.*, 1983).

One route of rabies vaccine developments has involved the incorporation of soluble glycoprotein into liposomes (synthetic phospholipid vesicles; Oth *et al.*, 1987; Sureau and Perrin, 1989), or in ISCOMS (immunostimulating complexes; Morein *et al.*, 1984; Morein and Simons, 1985). In both procedures a good CTL response is observed and the correct native protein structure ensures effective induction of neutralizing antibodies. Rabies glycoprotein presented in lipid vesicles failed to induce a CTL response (Wunner *et al.*, 1983). Oth *et al.* (1987) anchored purified G onto unilamellar liposomes resulting in a structural similarity of G to that observed in virus, and promoting an IL-2 response that was not observed by immunizing with rabies G alone. Morein and coworkers (1984) presented rabies glycoprotein on Quil A-based ISCOMS and have observed good immune responses. Rabies G/ISCOMS protected dogs and mice against street rabies virus challenge (Fekadu *et al.*, 1992). In addition to acting as a carrier/presentation molecule for rabies G, the ISCOM has demonstrated its usefulness as a strong adjuvant in some veterinary vaccines (Morein *et al.*, 1985). An IM immunization of mice with plasmid DNA containing the rabies glycoprotein sequence stimulated VNA and protected mice against challenge (Xiang *et al.*, 1994).

Analogous to G presentation on vesicles or complexes is the use of eukaryotic cell surfaces as a system of antigen presentation. Glycoprotein sequences were expressed off the polyhedrin promoter of *Autographa californica* nuclear polyhedrosis virus (AcNPV) in infections of insect cells (Préhaud *et al.*, 1989b). High neutralizing antibody titers and protective immunity were demonstrated by injecting mice intraperitoneally (IP) or intramuscularly (IM) with 10^6 - 10^7 whole insect cells expressing rabies glycoprotein on the plasma membrane (Préhaud *et al.*, 1989b).

POX VIRUSES AS VACCINE VECTORS FOR RABIES GLYCOPROTEIN

The development of live "hybrid" or "recombinant" viruses and their inoculation into a host species permits the development of an immune response to the virus as well as to any inserted and expressed "foreign" sequences (Fields and Chanock, 1989). The recombinant virus can be modified to reduce its own virulence as well as to accommodate foreign sequences. Immunization with a live viral vector enables an efficient activation of all phases of the immune system, although results in potential drawbacks such as the genetic instability of the virus, the presence of contaminating viruses, and persistent infection (Murphy and Chanock, 1985). The development of recombinant viral vectors has been carried out primarily for DNA viruses such as vaccinia and adenovirus (Brown, 1989, 1990), and has been applied to the expression of rabies glycoprotein (Kieny *et al.*, 1984; Prevec *et al.*, 1990).

Vaccinia virus (VV) vectors expressing rabies glycoprotein sequences have proven promising in inducing immune responses and offering protection against a challenge with rabies virus (Kieny *et al.*, 1984; Wiktor *et al.*, 1984; Blancou *et al.*, 1986; Rupprecht *et al.*, 1986, 1988; Tolson *et al.*, 1987, 1988; Koprowski, 1988; Artois *et al.*, 1990; Desmettre *et al.*, 1990; Thomas *et al.*, 1990; Brochier *et al.*, 1991). The introduction of foreign sequences into the vaccinia thymidine kinase gene reduces vaccinia virus virulence significantly (Buller *et al.*, 1985). Kieny *et al.* (1984) developed a recombinant vaccinia virus vaccine expressing rabies ERA strain glycoprotein under control of the vaccinia virus 7.5K early-late promoter (VVTGgRAB). The vaccine was effective and safe in rodents, foxes, and raccoons with no generalized reactions but mild cutaneous inflammations were observed. Laboratory rodents and isolated raccoons were demonstrated to have a rapid induction of virus neutralizing antibodies that protected against challenge with lethal rabies virus (Wiktor *et al.*, 1984; Rupprecht *et al.*, 1988). Foxes survived a viral challenge one year after oral vaccination (Kieny *et al.*, 1984). Thomas *et al.* (1990) looked at the primary multiplication site of VVTGgRAB after oral administration to foxes: the recombinant virus was detected by viral isolation and indirect immunofluorescence in tonsils, buccal mucosa, and soft palate 24 hours after inoculation. High immunogenicity of the vaccine was reported for raccoons administered 10^8 pfu/ml of V-RG in a sponge bait, or by intradermal or intramuscular routes (Rupprecht *et al.*, 1988). No VV was detected in the salivary glands of

raccoons immunized by the oral route, although a low level of V-RG transmission between male-female pair combinations of raccoons as measured by VNA development was detected (Rupprecht *et al.*, 1988). Groups of cattle were administered V-RG; no horizontal transmission of the vaccine was obtained and all vaccinated animals showed significant antibody titers of at least 500 $\mu\text{g}/\text{mL}$ at 30 days post immunization (Koprowski, 1988). Brochier and coworkers (1991) utilized the vaccine in a field trial: 25,000 baits containing the vaccine along with a tetracycline marker were air-dropped into a 2,200 km^2 region in southern Belgium. The researchers report that 81% of sampled foxes developed immunity in response to the vaccine. No cases of rabies disease were detected in either foxes or domestic animals as late as 24 months post-distribution. The safety and lack of residual pathogenicity by V-RG for wild rodents were demonstrated by oral or mucosal immunization of animals from four genera (*Apodemus*, *Arvicola*, *Clethrionomys*, and *Microtus*) (Artois *et al.*, 1992).

Although this vaccine was effective, Anderson (1991) presents several problems with the recombinant vaccinia-virus vaccine that may affect whether or not the vaccine would be practical to use. The cost of preparing such a vaccine would be similar to that incurred by a fox culling program, but would be too expensive for implementation in poorer countries. In addition, a field study carried out in an interepidemic period would result in lower predicted numbers of rabies infections. Müllbacher *et al.* (1989) observed the ineffectiveness of stimulating mucosal immunity with vaccinia virus. The recombinant virus has shown poor growth *in vivo* with resulting low levels of antigen production. Vaccinia virus Copenhagen or WR variants were used to develop the rabies vaccines; both variants increased the side effects in humans, such as post vaccination encephalitis or progressive VV infections (Esposito *et al.*, 1988; Kaplan, 1989; Hruby, 1990). Finally, insufficient research has been carried out on poxvirus ecology such as interactions of recombinant vaccinia viruses with wild poxviruses that are present in target and non-target species (Kaplan, 1989).

Alternative mammalian poxviruses, replication restricted in human hosts, have been evaluated as potential vectors (Hruby, 1990). Esposito *et al.* (1988) constructed raccoon poxvirus recombinants expressing rabies glycoprotein off either the $P_{7,5}$ early-late class promoter or the P_{11} late class promoter. High VNA levels were observed in raccoons and low VNA were seen in dogs but only one out of three skunks developed neutralizing antibodies (Esposito *et al.*, 1988).

Lodmell *et al.* (1991) evaluated raccoon poxvirus recombinants expressing either the rabies nucleoprotein (RCN-N) or the glycoprotein (RCN-G). Only the RCN-G vector stimulated neutralizing antibody production although RCN-N could protect A/WySnj mice against a lethal challenge. Canarypox virus was also utilized to express rabies glycoprotein (Taylor and Paoletti, 1988; Taylor *et al.*, 1991; Cadoz *et al.*, 1992). The safety and efficacy of recombinant canarypox-RG vector, ALVAC-RG, was demonstrated in animals (Taylor and Paoletti, 1988), and in a phase 1 clinical trial of 9 volunteers (Cadoz *et al.*, 1992).

VESICULAR STOMATITIS VIRUS

Vesicular stomatitis virus is considered a prototype rhabdovirus and has served as a model system for research. Because of the ease of working with VSV and its rapid rate of growth, the virus system was used by many laboratories to study the transport of glycoproteins from the site of synthesis in the cytoplasm to the plasma membrane surface. Glycoprotein synthesis and processing studies carried out with VSV apply equally to rabies glycoprotein.

Disease And Vectors

Vesicular stomatitis virus (VSV) serotypes Indiana (Ind) and New Jersey (NJ) are both considered prototype VSV strains (Cartwright and Brown, 1972; Tesh *et al.*, 1983). Both serotypes are prevalent in North, Central, and South America infecting a broad host range of species including mammals, mosquitoes, sandflies, and houseflies (reviewed in Shope and Tess, 1987). Epizootic outbreaks of VSV Indiana and NJ are observed in the USA in predominant 10 year cycles although smaller outbreaks occur yearly during the summer (Jonkers, 1967). Outbreaks are rapid and usually affect cattle within 48 hours after contact (Jonkers, 1967). VSV is of veterinary importance causing vesicular stomatitis, a disease characterized by fever, lethargy, vesicular lesions, mastitis, and decreased milk production (Alderink, 1984). VSV disease contributes to financial losses in dairy cattle herds (Alderink, 1984). In humans, primarily the veterinary and farming populations, VSV Indiana or NJ contamination of cuts or

scratches in the skin can cause an acute, influenza-like disease lasting 3 to 6 days (Tesh and Johnson, 1975).

Pathogenesis

The pathogenesis of a vesiculovirus infection depends on several factors including dose of virus, route of infection, species infected, MHC haplotype, and age of the species (Shope and Tesh, 1987). In animal studies, an intracerebral or intranasal inoculation into rodents results in a lethal infection (Murphy *et al.*, 1975), while an intraperitoneal injection leads to a persistent infection (Bruno-Lobo *et al.*, 1968a, 1968b). Newborn rodents are susceptible, while older rodents inoculated by a subcutaneous route recover and develop protective antibodies. The correlation between newborn rodent susceptibility and route of inoculation is not yet explained. Intranasal infection of BALB/c-H-2^{dm2} mice with VSV results in initial virus replication in the nasal cavity followed by movement of virus simultaneously into the lungs and olfactory bulb (Forger *et al.*, 1991). Overall, low levels of replication and minimal pathology were evident in the lungs although brain tissue, especially ventricles and lumbosacral regions of the spinal cord, contained severe pathology (encephalitis, necrosis, and mononuclear infiltrate).

Vaccine

At the present time, there is no licensed vaccine against vesiculovirus. Olitsky and coworkers (1928; as reported by Shope and Tesh, 1987) carried out IM immunizations of cattle against VSV and observed immunity against a local challenge. Attempts to vaccinate against VSV were also made using inactivated virus, purified subunits of virus, or virus recombinants expressing VSV glycoprotein (Holbrook and Geleta, 1957; Lauerman, 1967; Mackett *et al.*, 1985) as it was shown that the glycoprotein is a major target of neutralizing antibodies (Kelley *et al.*, 1972).

VSV GLYCOPROTEIN

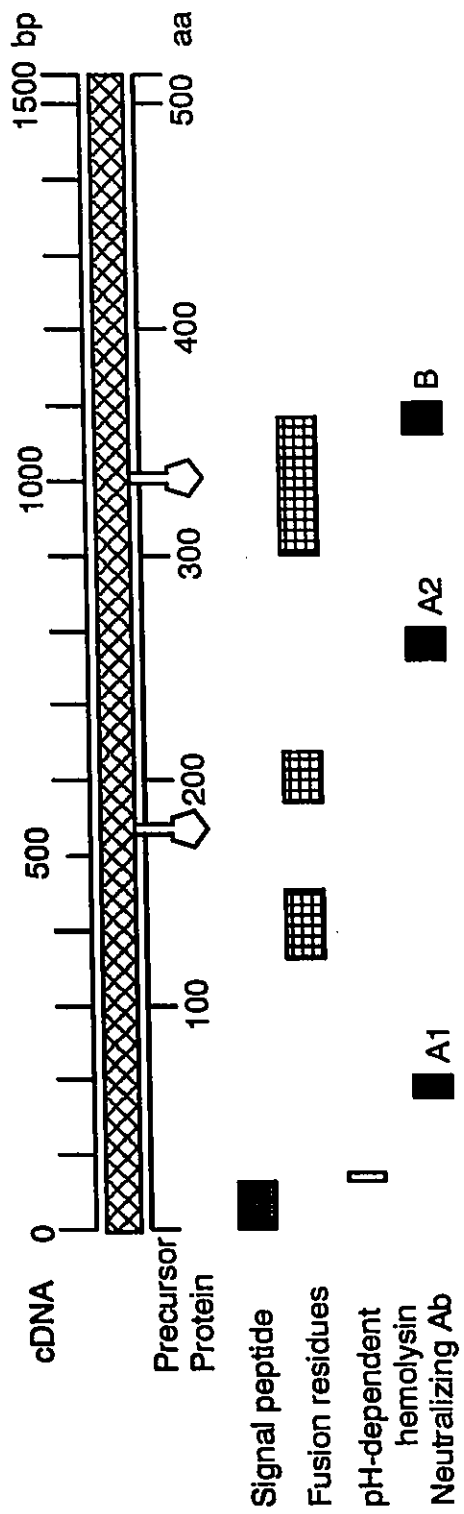
Characteristics

The coding sequence for VSV Indiana glycoprotein (G) is 1665 bases translating into a 511 aa mature protein with a M_w of 63,000 daltons (Fig. 1.3). The precursor protein contains a 16 aa NH_2 -terminus signal sequence cleaved after protein translation. VSV Indiana and New Jersey strains share a 50.9% homology (Gallione and Rose, 1983). The Indiana glycoprotein contains two N-glycosylations (at Asn 194 and Asn 351 with respect to the precursor protein sequence) resulting in the synthesis of mature protein G2 with a M_w of 69,000 on SDS-PAGE (Etchison and Holland, 1974; Toneguzzo and Ghosh, 1977). A minimum of one oligosaccharide addition is required for virus transport to the cell surface (Gibson *et al.*, 1979; Machamer *et al.*, 1985). VSV G is found as a trimer on the virion surface (Doms *et al.*, 1987). Further processing involves the esterification of 1-2 palmitate groups to the glycoprotein on the NH_2 -terminal side of the transmembrane domain; the acyl groups have a role in protein stabilization (Schmidt and Schlesinger, 1979; Rose and Gallione, 1981). Nonacylated glycoprotein is transported to the cell surface but fails to be incorporated into virions (Lodish and Kong, 1983).

Shortened versions of VSV G comprising 71 amino acids of the carboxy terminus can be detected in purified virion as well as in membranes of wt VSV-infected cells (Chen *et al.*, 1988). The 9 kD protein, known as Ga2, contains 22 amino acids of the ectodomain and is acylated with palmitic acid indicating efficient transport through the Golgi. VSV-infected cells secrete a soluble protein, G_s , lacking the transmembrane and cytoplasmic domains (Kang and Prevec, 1970; Grünberg *et al.*, 1991). In cells transfected with truncated G cDNA (lacking the carboxy-terminal 79 aa), the anchorless G is glycosylated and secreted at slower rates (Rose and Bergmann, 1982) indicating that the ectoderm is sufficient for a transport-competent protein (Schmidt *et al.*, 1992). The function of truncated G_s is not known although Chen *et al.* (1988) propose that the virus may generate soluble G_s antigen which, in conjunction with virions containing truncated Ga2, would act as an escape mechanism from the immune system.

FIGURE 1.3: VSV INDIANA GLYCOPROTEIN

Schematic representation of VSV (Indiana) glycoprotein and its immunogenic determinants. The 1665 nucleotide sequence is translated into a 511 amino acid mature protein. Glycosylation sites at asparagine residues 194 and 351 are represented by inverted tree structure. Functional domains of the VSV (Indiana) glycoprotein include fusion domains (residues 136 to 166, 206 to 226, and 316 to 376), hemolysin activity domains (residues 17 to 22), and domains important for the induction of neutralizing antibodies (residues 53-55, 257-263, and 357-367). All referrals to residue positions are based on the precursor protein sequence.



Co-Translational And Post-Translational Processing Of VSV Glycoprotein

VSV glycoprotein is used as a model system to examine post-translational protein folding, oligomeric assembly, transport, and processing of viral membrane proteins (reviewed by Doms *et al.*, 1993). Protein folding commences co-translationally as the newly synthesized molecule enters the endoplasmic reticulum (ER) and can be monitored in pulse-chase experiments by examining disulphide bond formation, detection of conformational epitopes, and protease resistance. Each domain within the protein is folded independently. The folding half-time of VSV G is approximately 3 min while the $t_{1/2}$ for assembly into oligomers is 7 min; both steps are rate-limiting for transport from the ER (Balch *et al.*, 1986; Kreis and Lodish, 1986; Doms *et al.*, 1987; De Silva *et al.*, 1990). Folding and disulphide bond formation are attained with the assistance of "molecular chaperone" proteins which bind polypeptides and prevent aggregation; these chaperones include protein disulphide isomerase (PDI) and peptidyl prolyl isomerase (PPI) (reviewed by Doms *et al.*, 1993). Gallione and Rose (1985) using a temperature-sensitive (*ts*) strain of VSV, *tsO45* (containing a Phe for Ser 220 substitution in the ectodomain), observed a lack of glycoprotein transport to the cell surface at non-permissive temperatures. *tsO45* glycoprotein was misfolded and remained aggregated (Doms *et al.*, 1987; De Silva *et al.*, 1990) or in a stable association with heavy chain binding protein BiP in the ER (Machamer *et al.*, 1990). Doms *et al.* (1988), in studies of 12 VSV G proteins with mutations in the ectoderm, transmembrane domain, or cytoplasmic domains, concluded that ectodomain sequences were critical for protein folding and trimerization; mutations in other domains had little effect on folding but either decreased the rate of or blocked the transport process - this was confirmed in mutagenesis studies by Zagouras *et al.* (1991). Deletions in the cytoplasmic domain affect the intracellular transport of VSV G protein by blocking transport or reducing the rate of oligosaccharide processing (Rose and Bergmann, 1983; Crise *et al.*, 1989; Zagouras *et al.*, 1991). Either membrane proximal or membrane distal portions of the cytoplasmic domain are required for efficient assembly of glycoprotein into virions during virus budding (Whitt *et al.*, 1989).

During translation a high-mannose precursor oligosaccharide (glucosamine₃-mannose₅-N-acetylglucosamine₂) is transferred from the carrier lipid dolichol to asparagine residues on the

nascent chain in the ER (Rothman and Lodish, 1977; Rothman *et al.*, 1978). Glycosylation is blocked in the presence of tunicamycin which inhibits the formation of N-acetylglucosamine-dolichol-phosphate (Tkacz and Lampen, 1975). The acceptor asparagine is found in the tripeptide sequence Asn-X-Thr/Ser, where X is any amino acid except for aspartic acid and proline. The trimming of glucosamine and mannose residues occurs in the ER and in the cis-Golgi ($t_{1/2}$ 15-20 min; Zagouras *et al.*, 1991). Endo- β -N-acetylglucosaminidase H (Endo H) cleaves N-acetylglucosamine residues of high-mannose oligosaccharides (Tarentino and Maley, 1974; Robbins *et al.*, 1977). Endo H resistance is acquired in the medial Golgi when all glucosamine residues and most of the mannose residues are removed, and galactose, N-acetylglucosamine, and sialic groups are added to the core structure (Robbins *et al.*, 1977; Tabas *et al.*, 1978). The final structure of the oligosaccharide groups is complex and consists of three chains of N-acetylglucosamine/ galactose/sialic acid attached to a mannose-3-N-acetylglucosamine-2 (Man3-GlcNAc2) core (Hubbard and Ivatt, 1981).

In tunicamycin-treated cells infected with VSV (Indiana) San Juan, nonglycosylated protein aggregates in the ER resulting in severely decreased levels of virus production yet the specific infectivity of the virus remains comparable to wild type (Leavitt *et al.*, 1977a, 1977b; Gibson *et al.*, 1978). From these results, researchers proposed two roles for protein glycosylation: to facilitate assembly of VSV particles and to protect the virion from proteases. By transfecting cells with cDNA encoding G protein with altered consensus sequences for N-linked glycosylation it was shown that glycosylation at either one of the potential glycosylation sites was sufficient for protein transport to the cell surface, and by eliminating both consensus sequences no protein transport was detected (Machamer *et al.*, 1985). Machamer and Rose (1988a) introduced glycosylation sites into G cDNA lacking the two normal consensus sites (Asn-X-Ser/Thr): six of the eight introduced sites were glycosylated but only two of the six promoted transport of the G protein - one site at amino acid 133 (mutant QN1/TA1,2; Asn substituted for Gln) and the other at amino acid 233 (mutant EN2/TA1,2; Asn substituted for Glu). Glycosylation may act as a signal for transport to the cell surface, or may promote conformational changes required for protein transport (Machamer *et al.*, 1985; Machamer and Rose, 1988a, 1988b).

Glycoprotein Binding Of Cell Surface Receptor

As is common for rhabdoviruses, the VSV glycoprotein is used as a virus receptor ligand (Cartwright *et al.*, 1970; Bishop *et al.*, 1975). The precise cellular receptor for vesicular stomatitis virus has not been isolated though basic properties concerning VSV attachment have been studied. Protease treatment of virions removes the surface glycoprotein and reduces the infectivity by more than 10^5 fold (Cartwright *et al.*, 1969). L cells treated with neuraminidase, removing sialic groups, were able to adsorb VSV resulting in infection (Schloemer and Wagner, 1975a, 1975b; Wagner, 1987). Trypsin treatment of L cells (Schloemer and Wagner, 1975b) or Vero monkey cells (Schlegel and Wade, 1983) has little effect on virus attachment. The cellular binding of VSV is pH-dependent (optimum pH 6.5) (Matlin *et al.*, 1982). At 37°C, bound virus is endocytosed by a clathrin-mediated pathway to become protease resistant ($t_{1/2}$ of 30 min); at this time, virus may be observed localized in coated pits on the cell surface as well as in coated vesicles and larger smooth surfaced vesicles (Matlin *et al.*, 1982; Rigaut *et al.*, 1991).

Role Of Glycoprotein In Fusion And Hemolysis

VSV glycoprotein is responsible for cell-to-cell fusion *in vitro* (Mifune *et al.*, 1982; Florkiewicz and Rose, 1984; Woodgett and Rose, 1986) and erythrocyte hemolysis (Mifune *et al.*, 1982). Upon transport of virus to lysosomes, the low pH of the compartment (pH 5.5-6.5) triggers viral envelope and lysosomal membrane fusion and results in nucleocapsid and matrix protein transfer to the cytoplasm (Bailey *et al.*, 1989; Rigaut *et al.*, 1991). Florkiewicz and Rose (1984) using a stable cell line with integrated glycoprotein cDNA found that glycoprotein expression promoted cell fusion forming polykaryons at pH 5.5, a reaction preventable by the addition of anti-glycoprotein MAbs. Fusion was observed at a pH 0.5 units lower than that seen for cells infected with VSV virus. The authors propose that three hydrophobic domains (residues 136-166, 206-226, and 316-376) may be important for fusion. Schlegel and Wade (1985) identified the amino-terminus six residues of the mature Indiana G as containing a pH-dependent hemolysin, an activity of the synthetic peptide correlating with the presence of a Lys residue in position one. Woodgett and Rose (1986) expressed G with a substitution of the terminal Lys and

observed pH-dependent fusion activity comparable to the wild type glycoprotein concluding that hemolytic activity was not related with the fusion activity. Whitt *et al.* (1990) isolated mutant QN-1 with an additional N-linked glycosylation at residue 133 near 19 uncharged aa (residues 134-152) that are conserved between serotypes; the resulting virus was noninfectious failing to undergo membrane fusion activity. In contrast, Bailey *et al.* (1989) have demonstrated that glycosylation of Ind G is not required for fusion activity in insect *Spodoptera frugiperda* cells.

VSV GLYCOPROTEIN AND THE IMMUNE RESPONSE

In encountering the immune system, the glycoprotein acts as a B cell mitogen (Goodman-Snitkoff *et al.*, 1981), and is responsible for both neutralizing antibody (Kelley *et al.*, 1972; Leifrançois and Lyles, 1982a, 1982b, 1983a; Volk *et al.*, 1982; and others) and CTL responses against the virus (Kelley *et al.*, 1972; Zinkernagel *et al.*, 1978a, 1978b; Hale *et al.*, 1981; Yewdell *et al.*, 1986).

Induction Of Neutralizing Antibody

VSV glycoprotein is the target viral protein for antibodies that neutralize virus infectivity (Kelley *et al.*, 1972). Neutralizing antibodies are strain specific, *i.e.* there is no cross-neutralization between Indiana and New Jersey (Reichmann *et al.*, 1978). Researchers including Lefrançois and Lyles (1982a, 1982b), Volk *et al.* (1982), Seif *et al.* (1985), Vandepol *et al.* (1986), and Luo *et al.* (1988) have characterized epitopes on Indiana and NJ glycoproteins recognized by neutralizing and nonneutralizing antibodies.

Lefrançois and Lyles (1982a) evaluated VSV neutralizing epitopes in competitive binding assays with panels of MAbs reactive with either the Indiana or NJ strains of the virus. All neutralizing epitopes mapped to the viral glycoprotein: four nonoverlapping epitopes were defined on the NJ glycoprotein (A, B, C, D)_{NJ}, while five epitopes on the Indiana glycoprotein with varying degrees of overlap were characterized (A, B₁, B₂, C, D)_{Ind}. C_{Ind} was observed to overlap A_{Ind} and B_{1-Ind}, and to a degree, B_{2-Ind}. The majority of neutralizing MAbs were directed against group A_{Ind} suggesting this site to be immunodominant. Competitive binding assays further

divided A into A₁, A_{2a}, A_{2b}, and A₃ (Lefrançois and Lyles, 1983a). Studies of nonneutralizing epitopes were also done (Lefrançois and Lyles, 1982b): the nonneutralizing epitopes showed crossreactivity between the serotypes, and binding sites for neutralizing antibodies were demonstrated to be distinct from those binding nonneutralizing antibodies. Vandepol and coworkers (1986) characterized a panel of neutralizing and nonneutralizing MAbs to VSV Indiana by selecting for nonbinding mutants. The major neutralizing determinant, A, was contained in two discontinuous regions of the primary protein sequence, although the authors indicate that determinant A also contains continuous epitopes. Viral mutants selected for by A-specific MAbs 12, 13, and 18, were found to contain changes in the primary sequence at Val 53, Gly 54, and Thr 55 (corresponding to A₂), or at Asp 257, Asp 259, and Ala 263 (corresponding to A₁) indicating that the A determinant was discontinuous. A second neutralizing determinant, B, contained changes at residues Ser 357, Arg 358, Met 362, or Thr 367 (Fig. 1.3). The authors were unable to map epitope C due to a low frequency of mutant variants (Lefrançois and Lyles, 1983a). C_{Ind} probably overlaps with A_{2-Ind} as indicated by a reduced avidity for group C MAbs by A_{2-Ind} variants (Lefrançois and Lyles, 1983a).

In VSV NJ conformation dependent neutralizing epitopes are localized to the middle third of the glycoprotein between both glycosylation sites (at residues Asn 195 and Asn 305). Luo *et al.* (1988) sequenced NJ variants that resisted neutralization by MAbs directed against glycoprotein epitopes V, VI, VII, and VIII. Variants of epitope V contained a Glu 380 change to a Lys, epitope VI mutants had Pro 284 to Thr, epitope VII was defined by a change from Glu 226 to Lys, and epitope VIII variants had a Leu residue replacing Ser 293 (Luo *et al.*, 1988). Epitope V was shown by Luo *et al.* (1988) to be equivalent to the B_{Ind} epitope as well as to rabies neutralizing epitope III, a region associated with pathogenesis (Seif *et al.*, 1985). New Jersey epitope VI was in close proximity to Indiana A1, while epitope VII overlapped with rabies epitope II (Luo *et al.*, 1988). Grigera *et al.* (1991) evaluated the effect of glycosylation on epitope recognition and observed a 5- to 15-fold decrease in epitope recognition in the presence of tunicamycin. Recognition of neutralizing epitope IV, located in the amino terminus, was not affected by tunicamycin. The treatment of purified G protein isolated from VSV NJ virions with N-glycanase had little or no effect on recognition of deglycosylated G by neutralizing antibodies.

Grigera and coworkers (1991) concluded that the carbohydrate chains were required to stabilize the conformational epitopes.

Keil and Wagner (1989) constructed chimaeras and deletion mutants of VSV New Jersey and Indiana glycoproteins. By examining the ability of the mutants to bind to a panel of monoclonal antibodies, the researchers were able to map both neutralizing and non-neutralizing epitopes. The VSV NJ glycoprotein was found to contain four neutralizing epitopes, all mapping to the middle of the glycoprotein (residues 209-305). Neutralizing epitopes of VSV Indiana G were mapped to residues 96-199 (2 domains) and residues 302-444 (2 domains).

Disulphide bonds within the glycoprotein have a direct effect on correct conformational presentation and recognition of discontinuous epitopes. Extensive studies were carried out on VSV NJ glycoprotein using site-directed mutagenesis to convert individual cysteine residues to serines by site-directed mutagenesis (Keil and Wagner, 1989; Grigera *et al.*, 1992). Grigera *et al.* (1992) found that mutations of individual Cys 146, Cys 190, and Cys 209 residues resulted in the loss of neutralizing epitope VIII, while Cys 146 and Cys 124 were required for recognition of neutralizing epitope VII. Mutations in Cys 251, Cys 256, or Cys 289 affected recognition of both epitopes VII and VIII - these cysteines were found in the area mapped to contain these epitopes. From this work, the authors conclude that not only proximal but also distal cysteines determine the neutralizing epitopes of VSV NJ. Observations have been made that the sites of major neutralizing epitopes in VSV glycoprotein correspond to sites of carbohydrate attachment in the rabies glycoprotein.

CTL Epitopes

The importance of VSV glycoprotein in triggering a cytotoxic T lymphocyte response against VSV was established by several researchers (Hale *et al.*, 1978, 1981; Zinkernagel *et al.*, 1978a, 1978b; Lefrançois and Lyles, 1983b; Rosenthal *et al.*, 1983; Yewdell *et al.*, 1986). There are two types of CTL responses to VSV: a cross-reactive response lysing cells infected with either VSV_{Ind} or VSV_{NJ} viruses, and a VSV serotype-specific response (Rosenthal and Zinkernagel, 1980). Zinkernagel *et al.* (1978a) first observed cell-mediated immunity to VSV by measuring footpad swelling in mice locally inoculated with VSV. The cross-reactive CTL

response to cells infected with either NJ or Ind was demonstrated to be MHC class I-restricted (Hale *et al.*, 1978, 1981; Rosenthal and Zinkernagel, 1980), and was significantly inhibited after cells were treated with anti-MHC class I or anti-VSV antisera (Zinkernagel *et al.*, 1978a) but was not affected by serotype-specific MAbs (Rosenthal *et al.*, 1983). Zinkernagel and coworkers (1978b) used a *ts* mutant (*ts045*) to observe a correlation between the lack of glycoprotein production and a poor CTL response at nonpermissive temperatures. VSV variant *tI17*, expressing mutant glycoprotein, continued to induce a CTL response. The use of mutant *ts* G31 allowed some of the cytotoxicity to be attributed to the matrix protein - this was explained as a role of the matrix protein in maintaining glycoprotein conformation in the virion envelope (Zinkernagel *et al.*, 1978b).

Browning *et al.* (1990a, 1990b) have shown that soluble VSV glycoprotein can sensitize target cells for lysis by CD4⁺ CTL clones. The clones responded with proliferation and IL-2 release (Browning *et al.*, 1990b). CTL clones isolated from BALB/c-H-2^{dm2} mice with deletions of class I MHC H-2L^d were specific for Indiana G and failed to lyse cells infected with VSV NJ (Browning *et al.*, 1990a). In studies of VSV G presentation to class II-restricted VSV G-specific CTL clones, Reiss *et al.* (1992) showed that newly synthesized class II MHC chains are required for antigen presentation of both exogenously provided soluble glycoprotein and newly synthesized glycoprotein.

ADENOVIRUSES

Adenoviruses are intermediate-sized DNA viruses containing a linear double stranded genome ranging in size from 30 to 40 kbp (Fig. 1.4) (for excellent reviews consult Ginsberg, 1984; Horwitz, 1990). The virion is a nonenveloped icosahedron, approximately 70 nm in diameter, composed of proteins and DNA. The family Adenoviridae is divided into genus *Mastadenovirus* (infecting mammalian hosts), genus *Aviadenovirus* (isolated from birds), and a minor group infecting poikilothermic animals (such as the leopard frog) (reviewed in Ishibashi and Yasue, 1984) indicating a broad host range, although individual adenoviruses are host range and replication restricted. Of the over 100 known serotypes of adenovirus (Ad), 47 serotypes were isolated from human respiratory, conjunctival, ocular, or gastrointestinal sources (Straus,

1984; Hierholzer *et al.*, 1988). The human serotypes are grouped into six subgenera according to biochemical, immunological, and structural characteristics such as the ability to hemagglutinate rat or rhesus monkey erythrocytes (Rosen, 1960; Hierholzer, 1973), DNA homology (Green *et al.*, 1979a), restriction analysis, GC content (Piña and Green, 1965), and oncogenicity (Straus, 1984) (refer to Table 1.1). Member adenoviruses belonging to a subgenus have greater than 90% DNA homology, except for subgroup A viruses which share 48%-69% of sequences (Sussenbach, 1984). Initial descriptions of adenovirus oncogenicity were made for human Ad12 tumor induction in hamsters (Trentin *et al.*, 1962) although no malignancies due to adenovirus (Ad) have been reported in humans (Mackey *et al.*, 1976, 1979; Green *et al.*, 1979b). Hemagglutination properties of individual serotypes and higher GC content were observed to reflect the oncogenic potential of the virus (Huebner *et al.*, 1965).

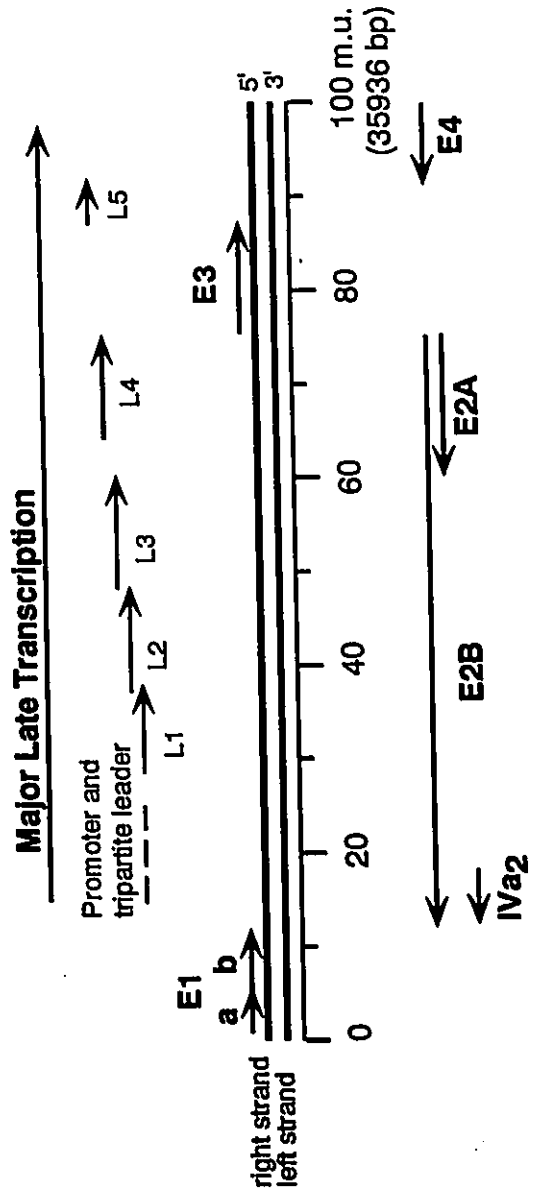
TABLE 1.1: CLASSIFICATION OF HUMAN ADENOVIRUSES

Subgroup	Serotypes	Tumors in Animals	G+C	Hemagglutination
A	12, 18, 31	High	48-49%	IV (little agglutination)
B	3, 7, 11, 14, 16, 21, 34, 35	Moderate	50-52%	I (complete)
C	1, 2, 5, 6	Low /none/	57-59%	III (partial)
D	8-10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42	Low /none/	57-61%	II (complete)
E	4	Low /none/	57-59%	III (partial)
F	40, 41	Unknown		III (partial)

(modified from Horwitz, 1990)

FIGURE 1.4: HUMAN ADENOVIRUS 5 GENOME

A schematic representation of the 35,936 kbp double-stranded adenovirus genome and its division into 100 map units (mu). Transcriptional regions are indicated: right-strand E1a (1.3-4.5 mu), E1b (4.6-11.2 mu), protein IX (9.8-11.2 mu), major late promoter (MLP) (at 16.5 mu) and five families of late mRNAs, and E3 (76.6-86.0 mu), while on the left strand IVa₂ (16.1-11.1 mu), E2A (67.0-61.5 mu), E2B (29-14.2 mu), and E4 regions (96.8-91.3 mu).



ADENOVIRUS DISEASE

Most of the identified human adenovirus serotypes exhibit a tropism for ocular and respiratory tissues resulting in endemic, epidemic, or sporadic infection cycles (reviewed in Straus, 1984). Human Ad types 1 through 24 were isolated from respiratory or ocular tissues while types 25 through 41 have been recovered primarily from stool samples. The majority of adenovirus-related illnesses are caused by Ad types 2, 1, 7, 3, and 5, in that order (Assaad and Cockburn, 1974), with the incidence of infection inversely correlating with age (Fox *et al.*, 1977). Symptoms commonly associated with adenovirus infections include coryzal illness (rhinorrhea, nasal congestion, and sneezing) or pharyngoconjunctival fever (fever, sore throat, conjunctivitis, mild illness) while lower respiratory tract infections with Ad3, 4, 7, or 21 may result in pneumonia (Straus, 1984). Endemic outbreaks of Ad1, Ad2, Ad5, and Ad6 are frequent in children: by three years of age, 80-90% of children have developed neutralizing antibodies to these viruses (Fox *et al.*, 1977; Straus, 1984). Ad epidemics are primarily due to Ad3, Ad4, Ad7, and Ad8. Epidemics of Ad4 and Ad7 are a noted cause of acute respiratory disease (ARD). ARD is predominant in military recruits undergoing basic training and affects up to 80% of recruits within the first three weeks of training - of these, 20% require hospitalization with such symptoms as headache, fever, chills, cough, sore throat, malaise, and anorexia (Straus, 1984; Ruben and Rorke, 1988).

CONTROL OF ADENOVIRUS

Vaccines against adenovirus types 4 and 7 were developed for military recruits with the intent of preventing acute respiratory disease (Chanock *et al.*, 1966; Edmondson *et al.*, 1966; Top *et al.*, 1971a, 1971b). Ad4 and Ad7 were grown in diploid cell culture using WI-26 or WI-38 cells; the virus was packaged into enterically-coated capsules which, when administered, allowed the virus to bypass the nasopharynx and upper respiratory tracts. Volunteers given Ad4 capsules developed virus neutralizing antibody (VNA) levels of 1:8 or greater as observed in 70-82% of individuals 3 weeks post-immunization (Chanock *et al.*, 1966). No evidence of virus transmission to non-immunized volunteers was detected (Chanock *et al.*, 1966). When

administered Ad4 capsules, army recruits stationed in a low ARD-risk camp developed VNA levels of 1:8-1:256 at one month post-immunization; on transfer to a camp with extensive seasonal outbreaks of ARD, almost all immunized men were protected against ARD while all recruits administered a placebo were infected with Ad4 (Edmondson *et al.*, 1966). Later studies by Top and coworkers (1971a, 1971b) using a combination of Ad4 and Ad7 in enteric-capsules confirmed the safety, potency, VNA induction, and lack of transmissibility of the dual vaccine.

ADENOVIRUS INFECTION AND REPLICATION IN CELLS

Adenovirus attachment to one of approximately 10^4 receptors present on the cell surface occurs by means of the fiber structure (Löndberg-Holm and Philipson, 1969). The virus/receptor complex migrates within the plasma membrane to clathrin-coated pits and is endocytosed (reviewed in Horwitz, 1990). A drop in the pH of the endosomal compartment initiates a change in the surface structure of the virus resulting in its release into the cytoplasm and transport by microtubules to the extremity of the nucleus. The viral DNA is passed into the nucleus for replication and transcription of the viral genome (Philipson *et al.*, 1968; Dales and Chardonnet, 1973). After polyribosome translation in the cytoplasm, polypeptides are transported into the nucleus for virion assembly (Velicer and Ginsberg, 1970).

The level of replication varies depending on the host cell type: high levels of virus are produced by cells permissive for adenovirus replication, whereas little or no virus is produced by cells nonpermissive for replication. Host range is determined by the presence or absence of cellular factors required for transcription or post-transcriptional processing (Löndberg-Holm and Philipson, 1969). Human adenoviruses grow efficiently in human cells but have reduced (semipermissive) or poor growth (nonpermissive) in other cell types. Semipermissive cells, such as the African green monkey kidney cells, may be blocked in the processing and translation of late viral protein mRNA or other steps along the viral synthesis pathway (Klessig and Anderson, 1975; Anderson and Klessig, 1984; Johnston *et al.*, 1985). Not all human cells support efficient adenovirus growth. Peripheral blood lymphocytes are nonpermissive (Horvath and Weber, 1988) lacking the appropriate receptor (Silver and Anderson, 1988), while lymphoblastoid cell lines (*e.g.* a Burkitt's lymphoma cell line) are semipermissive for adenovirus replication (Faucon *et*

al., 1982). Productive infections of hAd are observed in primary respiratory, ocular, and gastrointestinal epithelial cells (Fishaut *et al.*, 1980).

In a lytic infection, 10,000 virus particles are produced per cell along with an excess of viral protein and DNA (Green and Daesch, 1961). The replication cycle of adenovirus involves two phases, early and late, separated by the onset of viral DNA replication. Viral DNA replication begins 6 to 9 hrs post-infection (p.i.) (Horwitz *et al.*, 1973). Early in infection transcripts are made from the four separate early (E) regions: E1 (comprising E1A and E1B), E2, E3, and E4, and from the L1 region controlled by the major late promoter (MLP). The early-to-late switch is not thoroughly understood although *cis*-acting factors are involved (Thomas and Mathews, 1980). In permissive infections, adenoviral proteins gradually inhibit initiation of cellular DNA synthesis and block the transport of cellular mRNA precursors to the cytoplasm for translation (Hodge and Scharff, 1969; Piña and Green, 1969; McGuire *et al.*, 1972; Price and Penman, 1972). At 18 hours p.i., polyribosomes almost exclusively contain viral-specific mRNA (Raskas *et al.*, 1970; Lucas and Ginsberg, 1971; Beltz and Flint, 1979). Late in infection, viral transcripts accumulate at 10-fold higher levels than in the early phase.

Ad 2 AND Ad5 TRANSCRIPTIONAL REGIONS AND PROTEIN PRODUCTS

By convention, the AT (adenine/thymine)-rich half of the genome was designated the right-hand half; thus, DNA transcribed to the right is said to be transcribed off the right strand. The early transcriptional subunits map to both DNA strands of adenovirus (Fig. 1.4): early region (E) 1A, E1B, pIX, major late (ML), and E3 located on the right strand of the genome, and E2, IVa₂, and E4 situated on the left strand (reviewed by Ginsberg, 1984; Nevins, 1987). With the exception of pIX, each of the transcriptional subunits undergoes alternative splicing, and several subunits (E2, E3, and MLP) are characterized by multiple polyadenylation sites. Regulation of early protein synthesis occurs at the transcriptional level, and each unit varies in the onset and duration of transcriptional activity (reviewed in Berk, 1986a; Nevins, 1987): E1A is transcribed as an immediate early, followed by E1B, E2A, E3, and E4 (delayed early) transcription. IVa₂ and IX are classified as intermediate transcripts; both are synthesized in an E1A-independent manner after the start of viral DNA replication (Crossland and Raskas, 1983). Five families of

transcripts (L1 through L5) are detected off the major late promoter late in infection, yet only L1 transcripts are detected prior to viral DNA replication (Nevins, 1987).

Region E1

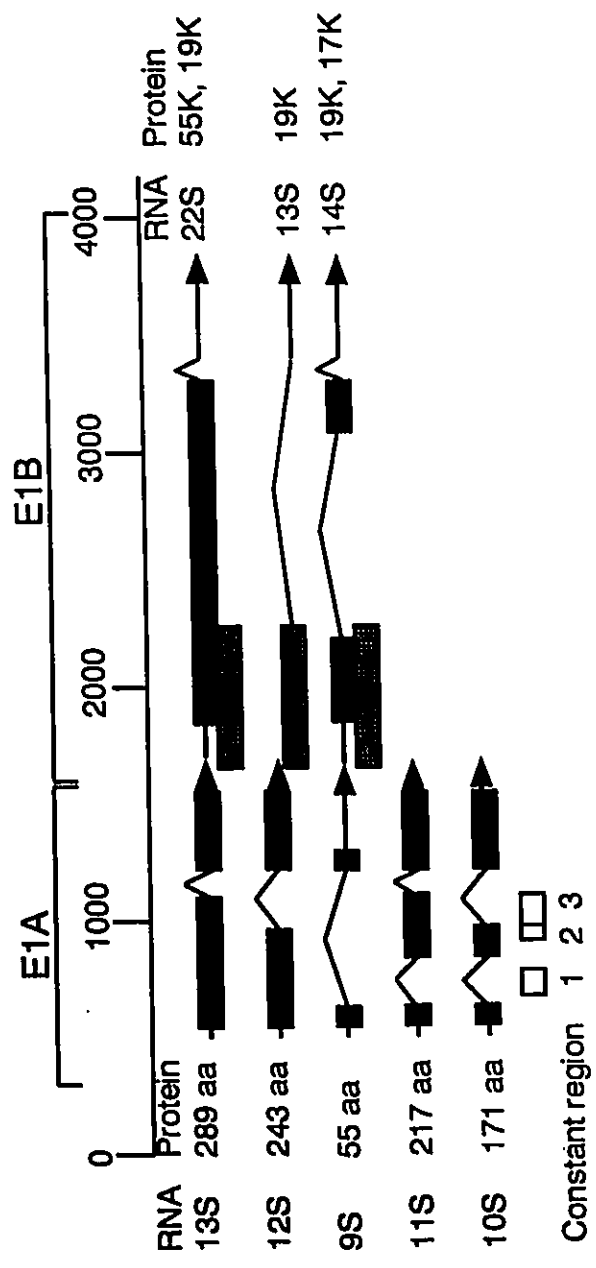
Early (E) region 1, located at map units (mu) 1.3-11.2, is transcribed from the leftmost part of the right strand. E1 contains three transcriptional units, E1A (mu 1.3-4.5), E1B (mu 4.6-11.2), and region IX which encodes virion structural protein IX (pIX) (mu 9.8-11.2) (Fig. 1.5).

Region E1A encodes at least three proteins synthesized from differentially spliced transcripts: 13S (32 kD), 12S (26 kD), and the 9S (13 kD) proteins, as well as two additional uncharacterized proteins. 13S and 12S are expressed throughout infection, while 9S is synthesized late in infection. Both 13S and 12S are acidic, nuclear phosphoproteins transported to the nuclei of infected cells by means of a C terminal transport sequence (Kripl *et al.*, 1986). Three regions of E1A are conserved between serotypes: two conserved regions, CR1 (residues 40 to 80) and CR2 (residues 121 to 140), are common to 13S and 12S, while CR3 (residues 141 to 80) is unique to 13S (Fig. 1.5). All three regions are associated with specific cellular functions (reviewed in Branton *et al.*, 1985; Berk, 1986b).

E1A proteins have a role in positive transcriptional (Nevins, 1981) or post-transcriptional control (Katze *et al.*, 1981). 13S is the primary transactivator of early and immediate early Ad or foreign transfected promoters (Jones and Shenk, 1979; Ricciardi *et al.*, 1981; Glenn and Ricciardi, 1985; Moran *et al.*, 1986; Green *et al.*, 1988). 12S was shown to be an inefficient activator (Leff *et al.*, 1984; Ferguson *et al.*, 1985). CR3, unique to 13S, is involved in the activation (Moran *et al.*, 1986; Lillie *et al.*, 1987; Schneider *et al.*, 1987; Jelsma *et al.*, 1988) likely through an interaction with promoter-specific factors (Berk, 1986a; Kovsdi *et al.*, 1986; Nevins, 1987). Berk (1986a) summarizes two theories for the transactivation: E1A interacts or modifies transcription factors to increase initiation frequencies by RNA polymerases II and III, or promotes the interaction of DNA with transcriptional factors. E1A transcriptional repression was also detected and the activity was mapped to CR2 (Borrelli *et al.*, 1984; Velchich and Ziff, 1985; Lillie *et al.*, 1987; Schneider *et al.*, 1987).

FIGURE 1.5: EARLY REGION 1 OF ADENOVIRUS TYPE 5

Schematic representation of E1 region. The E1 region is located at map units 1.3-11.2 of the adenovirus genome. E1A (mu 1.3-4.5) encodes three characterized proteins (13S, 12S, and 9S) and two uncharacterized proteins. Regions CR1 (aa 40 to 80), CR2 (aa 121 to 140), and CR3 (aa 141-180) are conserved between serotypes. E1B (mu 4.6-11.2) encodes 19K and 55K phosphoproteins. Region IX (mu 9.8-11.2) encodes virion structural protein IX. Lines represent spliced mRNAs. Black boxes represent E1A protein products, light grey boxes represent E1B 19K, while dark grey boxes represent E1B 55K and 17K protein products. Modified from White *et al.*, 1988.



E1A can immortalize primary cell culture (Houweling *et al.*, 1980), and in cooperation with E1B proteins or with activated oncogenes (such as *ras*) results in cellular transformation (Ruley, 1983; Graham, 1984b; Zerler *et al.*, 1986). The transformation efficiency is decreased with deletions of residues 4-25, 36-60, or 111-138 of exon 1 (Jelsma *et al.*, 1989), and CR3 is important for cellular transformation (Moran *et al.*, 1986; Zerler *et al.*, 1986; Whyte *et al.*, 1988b). Coimmunoprecipitation studies have revealed associations of E1A products with cellular proteins. Residues 111-127 bind the retinoblastoma gene product (*RBI*) (Egan *et al.*, 1988; Whyte *et al.*, 1988a) leading to oncogenic transformation (Egan *et al.*, 1988, 1989; Whyte *et al.*, 1988a). In quiescent cells E1A products induce mitosis, DNA synthesis, the synthesis of proliferating cell nuclear antigen cyclin (Zerler *et al.*, 1987), and the synthesis of epithelial growth factor (Quinlan *et al.*, 1987). Tumour necrosis factor α ($\text{TNF}\alpha$) sensitivity is induced by both 13S and 12S; the activity localizes to the amino-terminal portion of the CR1 domain, a region implicated in cellular immortalization, cell transformation, induction of DNA degradation and other functions (Moran and Mathews, 1987; Duerksen-Hughes *et al.*, 1991).

The E1B region produces the 19K (13S) and the 55K (22S) phosphoproteins (Persson *et al.*, 1982; Graham, 1984b; Stillman, 1986; McGlade *et al.*, 1987, 1989; White *et al.*, 1988). 19K can protect viral and cellular DNA during the infection process (Enzo *et al.*, 1981; Lai Fatt and Mak, 1982; Pilder *et al.*, 1984; White *et al.*, 1984). In cells infected with E1B 19K mutants, cellular DNA becomes fragmented, and cells exhibit abnormal CPE and undergo premature death as a consequence of E1A expression (Pilder *et al.*, 1984; Subramanian *et al.*, 1984a, 1984b; White *et al.*, 1984, 1988). In the presence of E1A proteins, E1B 19K represses E1A-dependent gene expression, while in the absence of E1A, 19K stimulates viral gene expression and DNA synthesis (White *et al.*, 1988). E1B 19K contains trans-activating functions for Ad early promoters (McGlade *et al.*, 1987); activation is upregulated in the presence of E1A 13S (Herrmann *et al.*, 1987).

The 19K has been reported to protect human cells against $\text{TNF}\alpha$ cytolysis (Gooding *et al.*, 1991a). Resistance to $\text{TNF}\alpha$ cytolysis was shown to be directly related to the property of primary cell transformation (as observed with BRK cells) suggesting that a single function was sufficient for both events (White *et al.*, 1992). In contrast, Vanhaesebroeck *et al.* (1990) failed to correlate $\text{TNF}\alpha$ sensitivity with E1 gene expression in various cell types (BRK, primary human

retinoblast, NIH3T3, and normal rat kidney cells). Both E1A and TNF α promote cytotoxicity, and E1B 19K probably functions in a similar manner to overcome this. E1B 55K contains protein kinase activity (Lassam *et al.*, 1979; Branton *et al.*, 1981) and is required for the shutoff of host cell synthesis (Babiss *et al.*, 1985) and for the transport and processing of Ad late mRNA (Babiss *et al.*, 1985; Pilder *et al.*, 1986a, 1986b; Williams *et al.*, 1986). Adenoviruses containing mutations in either the 55K or the 19K proteins fail to transform cells (Jones and Shenk, 1979; Fukui *et al.*, 1984; Graham, 1984b; Bernards *et al.*, 1986; Barker and Berk, 1987).

Major Late Promoter

The major late promoter (MLP), located at 16.5 mu of the adenovirus genome, is a strong promoter producing large amounts of structural proteins (reviewed in Horwitz, 1990). Messages transcribed off the MLP are differentially spliced and polyadenylated (Nevins and Darnell, 1978). MLP is active one hour post-infection resulting in the detection of a single class of late mRNA, the L1 family, in the cytoplasm (Shaw and Ziff, 1980; Nevins and Wilson, 1981). Transcription of L1 occurs both early and late in infection (Akusjärvi and Persson, 1981; Nevins and Wilson, 1981). After viral DNA replication is initiated, transcripts of all late classes of mRNA (L1, L2, L3, L4, and L5) are produced (reviewed in Sharp, 1984). Regulation was observed to occur at the level of polyA site selection by the polymerase. L1 polyA, although inefficiently recognized in infection, is preferentially used early in infection. Late in infection all five polyA sites are used (Prescott and Falck-Pedersen, 1992; Wiest *et al.*, 1992). Post-replication levels of transcription are increased 1000-fold, likely as a result of higher gene-copy numbers (Shaw and Ziff, 1980). MLP transcription is transactivated by E1A products (Nevins, 1981; Hen *et al.*, 1982; Lewis and Manley, 1985); in the absence of E1A, low levels of MLP transcripts are detectable (Nevins, 1981). All transcripts contain a tripartite leader spliced to the 5' termini. The leader sequences increase translational efficiency late in infection (Logan and Shenk, 1984). The structural proteins are described in APPENDIX 2.

Early Region 3

Early region 3, located between 76.6 and 86.0 mu of the right strand, encodes at least eight mRNA species (Hérissé *et al.*, 1980; Hérissé and Galibert, 1981; Sussenbach, 1984; Cladaras and Wold, 1985) (Fig. 1.6). All transcripts share a common 5' end (from nucleotides 27,568 to 27,939 of the Ad5 sequence) which splices to nucleotide 28,335 of the genome; the 3' ends of transcripts vary. Late in infection, the E3 region can be transcribed off the major late promoter (Chow *et al.*, 1977, 1979; Chow and Broker, 1978); the resulting mRNAs contain an additional exon known as the γ leader spliced to the major late promoter tripartite sequences. Six E3 products have been identified: gp 19K (Persson *et al.*, 1980a, 1980b), 14.7K (Tollefson and Wold, 1988; Wang *et al.*, 1988), 14.5K (Tollefson *et al.*, 1990a), 11.6K (Wold *et al.*, 1984), 10.4K (Tollefson *et al.*, 1990b), and 6.7K (Wilson-Rawls *et al.*, 1990) (Fig. 1.6). Research has determined that the E3 products, although not essential for viral replication *in vitro* (reviewed in Tooze, 1981) and in several animal model systems (Morin *et al.*, 1987; Ginsberg *et al.*, 1989), primarily function in host immunoregulation (reviewed in Wold and Gooding, 1991).

Adenoviruses have evolved two main lines of defense to escape immune recognition: first, the ability to escape from T cell-mediated immunity by downregulating major histocompatibility complex (MHC) molecules and antigen expression, and second, the ability to resist action by the cytokines TNF α and lymphotoxin (Oldstone, 1989; Müllbacher, 1992). Of the nine adenovirus E3 proteins identified to date, gp 19K, 14.7K, 14.5K and 10.4K function to prevent the destruction of virus infected cells (reviewed in Wold and Gooding, 1991; Müllbacher, 1992; Wold, 1992).

Gp 19K noncovalently binds to MHC class I heavy chain in cells transformed or infected with subgenera B, C, D, and E adenoviruses (Kvist *et al.*, 1978; Signäs *et al.*, 1982; Pääbo *et al.*, 1986a, 1986b) blocking post-translational processing and transport to the cell surface (Andersson *et al.*, 1985; Burgert and Kvist, 1985; Cox *et al.*, 1991; Lippé *et al.*, 1991). Adenoviruses of the A subgenus prevent MHC class I expression by reducing class I mRNA levels (Pääbo *et al.*, 1986a, 1986b). Mouse cells infected with wild type Ad or with a recombinant vaccinia virus expressing gp 19K down-regulate class I MHC and have a decreased sensitivity to lysis by alloreactive CTL (Burgert and Kvist, 1987; Cox *et al.*, 1990). Gp19K

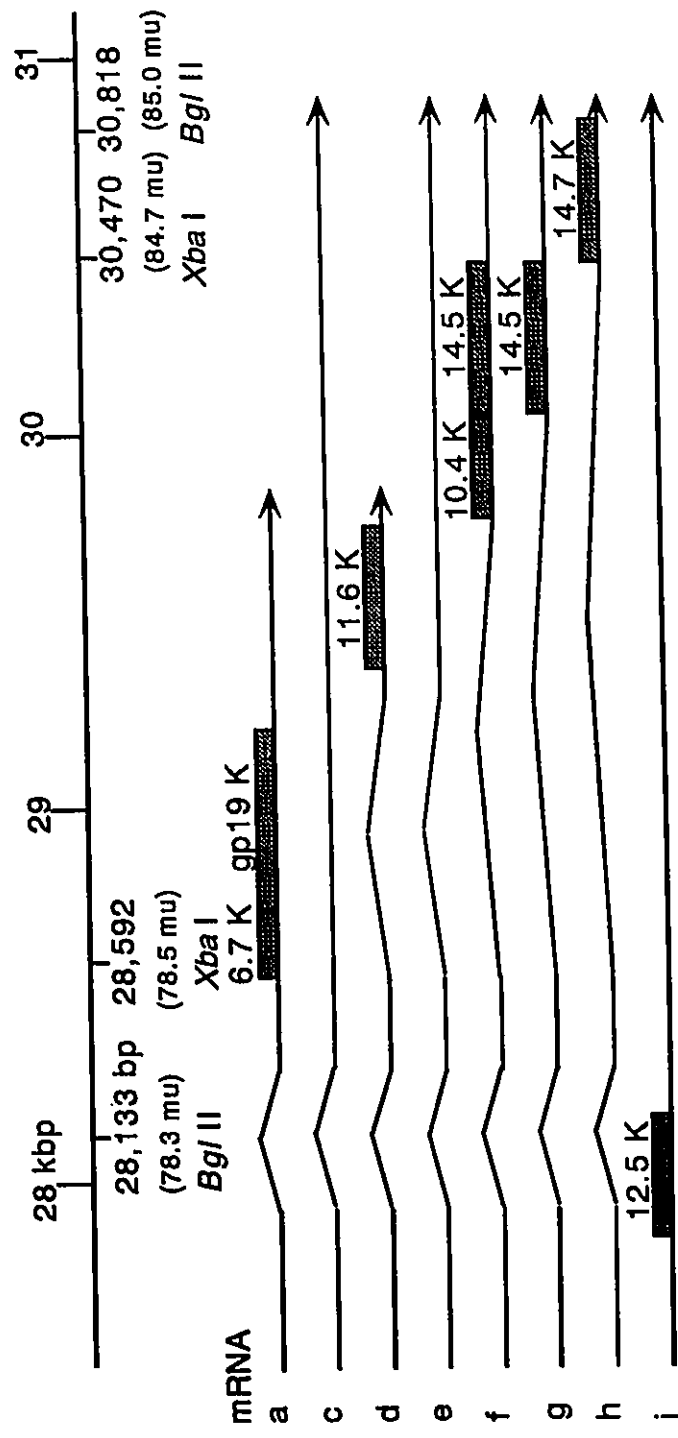
binding of mouse MHC is haplotype-dependent (Burgert and Kvist, 1987; Tanaka and Tevethia, 1988; Rawle *et al.*, 1989) and a higher affinity for human HLA-A2 antigen is observed (Severinsson *et al.*, 1986). The higher binding affinity may be interpreted that MHC haplotype may be a determinant of adenoviral persistence, although evaluations of ten human-derived cell lines showed normal levels of class I without any significant down-regulation by gp 19K (Routes and Cook, 1990). A subpopulation of human cells may exist whereby gp 19K can interact to limit the CTL response, and this may serve to explain the persistence, as opposed to a dissemination, of adenovirus in a small percentage of the population (Routes and Cook, 1990).

TNF α , produced by activated macrophages, is a polypeptide mediator with antiviral activity inhibiting the replication of RNA and DNA viruses (reviewed in Schütze *et al.*, 1992). The E3 14.7K protein protects most mouse cell lines against TNF α cytotoxicity including cells previously sensitized for lysis (Gooding *et al.*, 1988, 1990, 1991b; Horton *et al.*, 1990). 14.7K has no effect on the number of TNF α receptors present, nor on their ability to bind TNF α (Gooding *et al.*, 1990; Horton *et al.*, 1991). The amount of 14.7K expressed correlates with the extent of TNF α suppression, suggesting a physical interaction with components of the TNF α cytotoxicity pathway (Horton *et al.*, 1991). In most murine cell lines, excluding C3HA cells, complexed 10.4K/14.5K can also protect against cytotoxicity by TNF α (Gooding *et al.*, 1991b). The localization of 10.4K/14.5K to cytoplasmic membranes suggests a different mode of action against TNF α than would be predicted for 14.7K.

Epidermal growth factor receptor (EGF-R), a protein tyrosine kinase, is required for cellular DNA synthesis and mitosis. Shortly after adenoviral infection the 10.4K/14.5K complex reroutes the mature EGF-R to lysosomes resulting in its down-regulation (Carlin *et al.*, 1989; Tollefson *et al.*, 1991; Hoffman *et al.*, 1992). The down-regulation of the EGF-R is dose-dependent, and thereby indicative of a fairly direct interaction (Hoffman *et al.*, 1992). 10.4K/14.5K may also act on a later step of the EGF-R signal transduction pathway as 10.4K expression is sufficient for EGF-R down-regulation (Hoffman *et al.*, 1992). The ability of the 10.4K/14.5K complex to act on the EGF receptor may be related to the mechanism of action on TNF α .

FIGURE 1.6: EARLY REGION 3 OF ADENOVIRUS TYPE 5

Schematic representation of E3 region. E3, located between 76.6 and 86.0 mu of the adenovirus genome, encodes at least eight mRNA species. Transcripts share a common 5' end (nt 27,568 to 27,939) which is spliced to nt 28,335. Six E3 protein products have been identified: gp 19K, 14.7K, 14.5K, 11.6K, 10.4K, and 6.7K. Lines represent spliced mRNAs. Grey boxes represent E3 protein products.



An evaluation of murine cellular immune responses to Ad5 determined that an MHC-restricted CTL response was primarily directed against E1A proteins with a minor role by E2A and late virion proteins (Müllbacher *et al.*, 1989). E3 gene products interfere with E1A antigen expression (Zhang *et al.*, 1991) as determined by 8- to 10-fold increases in E1A levels and elevated degrees of lysability of L929 cells infected with E3 deletion mutants *dl327* or *dl355*. E1A mRNA levels were not affected suggesting that E3 products reduce E1A expression by a post-transcriptional mechanism to down-regulate the CTL response. The direct relationship between E3 products and E1A was demonstrated in transfections of 293 cells with plasmid expressing the E3 region (Zhang *et al.*, 1992); E3 protein products interfered with translation of viral mRNA molecules. The relationship is Ad-type specific and applies to subgenus C (Zhang *et al.*, 1992).

Other Transcriptional Regions

Regions E2, E4, and IVa₂ are transcribed in a leftward orientation (Fig. 1.2) (reviewed in Ginsberg, 1984; Nevins, 1987). The E2 region (E2A at 67.0-61.5 mu and E2B at 29-14.2 mu) contains two promoters: E2 early (75 mu) is active throughout infection, while E2 late (72 mu) is active after the initiation of replication (Berk, 1986a; Nevins, 1987; Horwitz, 1990). All E2 products, the 72K DNA binding protein, 120K polymerase, and 80K preterminal proteins, are required for viral DNA replication (Smart and Stillman, 1982; Friefeld *et al.*, 1983b). E2A 72K binds the ends of single and double-stranded DNA (Linné *et al.*, 1977), and is required for the elongation reaction during DNA replication (Horwitz, 1978; Klessig and Quinlan, 1982; Friefeld *et al.*, 1983a) and regulation of transcription (Nevins and Jensen-Winkler, 1980; Klessig and Quinlan, 1982; Friefeld *et al.*, 1983a). 72K functions in host range determination and assembly of infectious virus particles (Klessig and Grodzicker, 1979). The E4 region, located at 96.8 to 91.3 mu, contains seven open reading frames (Sharp *et al.*, 1974; Pettersson *et al.*, 1976). E1A 13S-dependent transcription of E4 products is short and is rapidly shut off (Gilardi and Perricaudet, 1984; Berk, 1986a; Nevins, 1987). Several E4 proteins have been identified: a 14 kD nuclear protein (Sarnow *et al.*, 1982; Downey *et al.*, 1983), 34 kD protein (Sarnow *et al.*, 1984), and a 19.4 kD product (Cutt *et al.*, 1987). The 34K product associates with E1B 55K

in the nucleus to regulate late gene expression (Sarnow *et al.*, 1984; Hemström *et al.*, 1988; Bridge and Ketner, 1990). The 19.4K, along with E1A proteins, transactivates E2 transcription by binding and activating the E2 promoter-specific cellular transcription factor E2F (Hemström *et al.*, 1991). Deletions of E4 affect viral DNA replication, and the shutoff of host cell protein synthesis (Halbert *et al.*, 1985).

The IVa₂ promoter (mu 16.1-11.1), active late in infection, initiates transcription from 211 bp upstream of the MLP start (Berk, 1986a). The IVa₂ and MLP share upstream regions which likely results in competition for RNA polymerase II and other transcription factors (Natarajan *et al.*, 1984; Natarajan and Salzman, 1985). IVa₂ synthesizes a 50K scaffold protein required for virus maturation (Persson *et al.*, 1979).

IMPLICATIONS OF DELETIONS IN THE ADENOVIRUS GENOME

To accommodate foreign protein sequences, regions of the adenovirus genome need to be deleted to meet the viral packaging requirement of 105% of the genome size. At least three regions are commonly used for the insertion of foreign DNA: the E1 and E3 regions, and a section of the genome between E4 and the right inverted terminal repeat (ITR) (reviewed in Graham and Prevec, 1991, 1992).

The E1 region is essential for virus replication and, when deleted, the resulting virus is helper-dependent for its replication function. The helper function is provided *in trans* by coinfection with wild type virus or, more commonly, by growing the recombinant virus on 293 cells, a cell line transformed with hAd5 DNA and expressing E1 gene products (Graham *et al.*, 1977). Sequences corresponding to the left ITR (1-103 bp) and packaging signals (194-300 bp) (Hearing *et al.*, 1987), and those corresponding to protein IX (from approx. 3500 bp) are required for packaging and viability and must be left intact (Ghosh-Choudhury *et al.*, 1977).

The limits of deletions in the E3 region are defined by coding sequences for structural proteins of the MLP L4 and L5 families. Two deletions of the E3 region were used to accommodate foreign inserts; both deletions, the *Xba*I and the *Bgl*II deletions, effectively eliminate sequences coding for the E3 proteins (Fig. 1.6). Only the E3 12.5K protein is produced by mutant virus carrying the *Xba*I deletion; 12.5K is synthesized in low amounts in wt-infected cells

and its role in infection is not clear. E3 proteins with roles in immunosurveillance, as reviewed in a previous section, would not be produced, and the potential consequences of E3 deletions should be considered. A decrease in Ad immunosurveillance would predict a decreased probability of viral persistence and less pathogenicity due to a more immediate and effective immune response against the virus. In fact, Ginsberg and coworkers (1989) in studies with the cotton rat (*Sigmodon hispidus*) observed that Ad mutants with deletions in E3, and specifically gp 19K mutants, promote significant increases in lymphocyte and monocyte/macrophage inflammatory responses. The inflammatory response commenced prior to the appearance of specific cytotoxic T cells indicating that the initial signal for macrophage infiltration came from cytokines released by activated macrophages present at the site of infection (Ginsberg *et al.*, 1989). The signal that triggers the inflammatory response is the cell-surface expression of the E1B 55K protein as shown by a decreased inflammatory response to E1B 58K mutants. Additional studies of Ad E3 mutants in a murine system were done to evaluate potential *in vivo* problems of vectors (Müllbacher, 1992). Ad E3 mutants were found to be highly virulent in mouse MHC haplotype strains that are susceptible to downregulation of class I MHC molecules and E1A products. Müllbacher (1992) proposed that E3 products modulate the host CTL response so as to limit the elimination of virus-infected cells; in the absence of E3 genes, the strong CTL response leads to immunopathology and death of the host, a response not favored in a vaccine. Effects of E3 deletions on the immunopathology of species other than the rodent family have not been established.

ADENOVIRUS AS A RECOMBINANT VIRUS VACCINE VECTOR

Advantages Of Recombinant Adenovirus Vectors

Human adenoviruses have been developed as expression vectors and as recombinant viral vaccines for mammalian species (reviewed in Berkner, 1988; Graham and Prevec, 1991, 1992). To date, human group B (serotype 7), group C (serotypes 2 and 5), and group E (serotype 4) adenoviruses have expressed viral, bacterial, and mammalian protein sequences (Table 1.2).

Numerous properties of adenoviruses lead to their suitability as vaccine vectors (Graham and Prevec, 1991, 1992). Group C viruses have been extensively characterized at the genetic and biochemical levels (Ginsberg, 1984). Thorough restriction enzyme maps of serotypes 2 and 5 were obtained enabling the selection of rare restriction sites that could be used during DNA manipulation (for hAd5 these include *Bam*HI, *Eco*RI, and *Xba*I). Ad DNA can be incorporated into a bacterial plasmid which, when transfected into 293 cells, regenerates infectious virus (Graham, 1984a; Ghosh-Choudhury *et al.*, 1986). Expression levels of foreign sequences can be dictated by Ad promoters or by the introduction of exogenous promoters. Adenovirus recombinants are highly stable maintaining foreign sequences over serial passage if the packaging capacity (105%) is not exceeded (Bett *et al.*, 1993). Berkner *et al.* (1987) have also observed lower yields of recombinant virus when recombinant size approached the packaging limit. In infected cells permissive for adenoviral replication, up to 10,000 plaque-forming units (pfu) of recombinant virus can be produced; the virus remains concentrated in the cell permitting an efficient harvest. Group B, C, and E serotypes are considered non-tumorigenic. Finally, the safety and efficacy of adenovirus as a vaccine has been conclusively demonstrated with Ad4 and Ad7 enteric immunizations of humans. With a packaging capacity of 105%, only an additional 2-3 kb of foreign sequences can be introduced into the genome which may limit the insert choice. The deletion of regions nonessential for virus replication increases the capacity for foreign sequences.

Construction of Recombinant hAd5 Vectors

The insertion of foreign sequences into the adenovirus genome is a multistep process (Berkner, 1988; Graham and Prevec, 1991, 1992). In protocols developed by Graham and fellow researchers (Graham and van der Eb, 1973; Graham *et al.*, 1977; Haj-Ahmad and Graham, 1986) the foreign sequences, with/out exogenous promoters and polyadenylation sequences present, are first cloned into a plasmid vector containing a subsection of the genome. The resulting construct is cotransfected into mammalian cells by calcium phosphate coprecipitation together with DNA corresponding to the remainder of the genome (Graham *et al.*, 1977), and virus is generated by

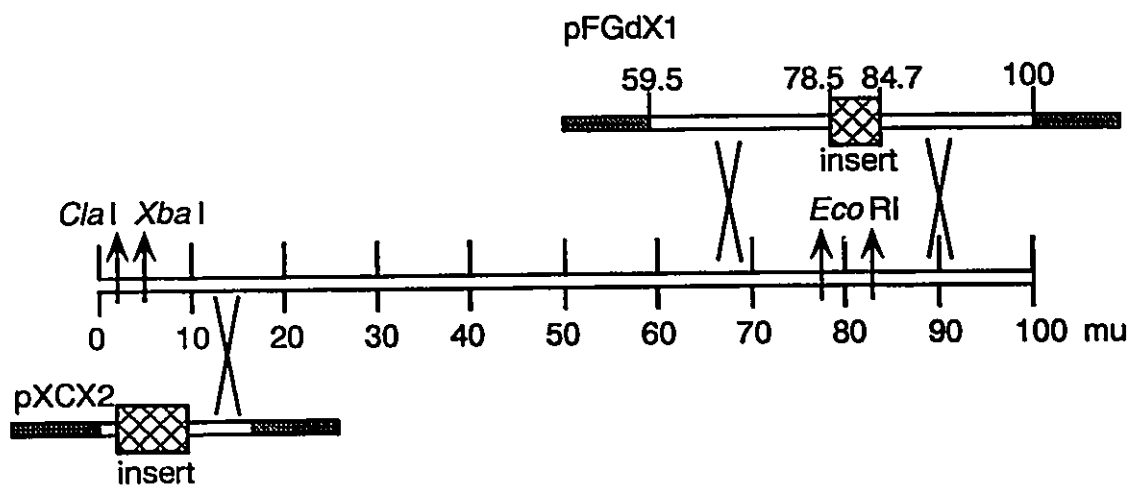
in vivo recombination (Fig. 1.7) (Arrand, 1978; Frost and Williams, 1978; Chinnadurai *et al.*, 1979).

By retaining sequences essential for packaging, the E1 region can accommodate deletions up to 3.2 kbp (Graham and Prevec, 1992). Inserts expressed in the E1 region are cloned into a plasmid vector such as pXCX2, a pBR322-based plasmid containing the left 16% of the hAd5 genome with a deletion of E1 sequences from 1.3-9.3 mu (Spessot *et al.*, 1989). The deleted sequences are replaced with *Xba*I recognition sequences or other convenient polylinker sequences to allow easy accommodation of inserts of up to 5 kbp in size. The construct may be cotransfected with virion DNA pre-cut with restriction enzymes specific for left end sequences, or cotransfected with plasmid pJM17 (McGrory *et al.*, 1988). pJM17 consists of the entire hAd5 genome along with 4.4 kbp of antibiotic sequences inserted at 3.7 mu of the E1 region; its total size exceeds the packaging limits of the adenovirus capsid which encourages high frequencies of recombination with the construct carrying the foreign insert. Cotransfections must be carried out in 293 cells to provide E1A helper functions *in trans*. Recombinants are verified by restriction analysis of viral DNA.

Foreign sequences to be rescued into the E3 region are first cloned into plasmid pFGdX1 (containing hAd5 sequences from 59.5 to 100 mu and a deletion of the *Xba*I fragment from 79.6 to 84.8 mu; Haj-Ahmad and Graham, 1986) or other derivative plasmids containing right-end hAd5 sequences (Bett *et al.*, 1993). The deletion of the *Xba*I fragment (1,878 bp) or the *Bgl*II fragment (78.3 to 85.8 mu; 2,685 bp) allows the introduction of approximately 4 or 5 kbp of foreign sequences, respectively. The construct is then cotransfected with *Eco*RI-restricted viral DNA and resulting plaques are screened for *in vivo* recombination. A disadvantage of this method is the high recovery rate of parental virus. To decrease the parental background one can cotransfect with plasmids such as pFG173 which contain a circular form of the viral DNA but with a lethal deletion in the E3 region (Graham and Prevec, 1992). The construction of double mutant recombinant viruses containing deletions of both E1 and E3 regions would generate a helper-virus-dependent (or 293 cell line-dependent) vector with a potential to contain over 8 kbp of foreign sequences.

FIGURE 1.7: INSERTION OF FOREIGN SEQUENCES INTO THE ADENOVIRUS TYPE 5 GENOME

A schematic representation of the recombination event between plasmids containing subsections of the genome to generate virus. The foreign sequences and additional exogenous promoters and polyadenylation sequences are cloned into a plasmid vector containing a subsection of the genome. The resulting construct is cotransfected into mammalian cells together with DNA corresponding to the remainder of the genome and virus is generated by *in vivo* recombination.



EXPRESSION OF FOREIGN INSERTS BY GROUP C ADENOVIRUSES

The expression of foreign inserts in hAd2 and hAd5 is determined by the presence of autologous (*i.e.* Ad-derived) promoters such as the E1A, E3, or major late promoters, or by exogenously-derived promoters such as the simian virus (SV) 40 early or human cytomegalovirus (HCMV) major immediate early promoter. Both of these viruses have been widely developed as recombinant vaccine vectors (Table 1.2).

Expression Of Foreign Inserts In The E1 Region

Synthesis of inserts in the E1 deletion is directed by strong exogenous promoters such as the hAd2 major late promoter (MLP) (Berkner and Sharp, 1984, 1985; Davis *et al.*, 1985; Berkner *et al.*, 1987; Alkhatib and Briedis, 1988; Alkhatib *et al.*, 1988; Eloit *et al.*, 1990; Lamarche *et al.*, 1990; Levrero *et al.*, 1991) or HCMV immediate early promoter (Boshart *et al.*, 1985; Jacobs *et al.*, 1992; Wilkinson and Akrigg, 1992). Transcription off the E1A promoter is rarely utilized (Ballay *et al.*, 1985; Karlsson *et al.*, 1986), and inserts should be placed in a leftward orientation to E1 to avoid aberrant mRNA production and the coexpression of other E1 genes (Berkner and Sharp, 1984; Davidson and Hassell, 1987; Alkhatib and Briedis, 1988).

The expression of inserts off the major late promoter inserted into the E1 region results in levels of protein expression as high as those obtained with the most abundant late Ad proteins (Berkner *et al.*, 1987). The translation efficiency of the inserted gene is dependent upon a minimal distance between the initiation codon and the tripartite leader (Berkner and Sharp, 1985; Berkner *et al.*, 1987). The first intervening sequence in the tripartite leader is included as it affects transcription initiation from the MLP in hAd2 and hAd5 (Mansour *et al.*, 1986; Cohen *et al.*, 1988; Jansen-Durr *et al.*, 1988). Vectors defective in the E1 region may express higher levels of foreign protein than nondefective vectors (Levrero *et al.*, 1991). This may be a direct result of diminished viral replication and decreased pathogenicity although Alkhatib and coworkers (Alkhatib and Briedis, 1988; Alkhatib *et al.*, 1988) obtained levels of measles

TABLE 1.2: RECOMBINANT ADENOVIRUS VACCINE VECTORS

Foreign insert	Orient.	Pro.	Comments	Reference
<u>Inserts into hAd4 or hAd7 E3</u>				
hepatitis B surface antigen	parallel	none	c, e, v/p	Lubeck <i>et al.</i> , 1989
hepatitis B surface antigen	parallel	none	c, e, multiple expression vector	Ye <i>et al.</i> , 1991
HIV-1 rev	parallel	none	c, e, multiple expression vector	Vernon <i>et al.</i> , 1991
<u>Inserts into hAd4 or hAd7 between E4 and right ITR</u>				
hepatitis B surface antigen	parallel	MLP	c, e, multiple expression vector	Ye <i>et al.</i> , 1991
hepatitis B surface antigen	parallel	MLP	c, e, v/dog	Mason <i>et al.</i> , 1990
HIV-1 envelope gp	parallel	MLP	c, e, multiple expression vector	Chanda <i>et al.</i> , 1990
HIV-1 gag, pro	parallel	MLP	c, e, E3 deletion present	Vernon <i>et al.</i> , 1991
<u>Inserts into hAd2 or hAd5 E1</u>				
chloramphenical acetyltransferase anti.	parallel anti.	MLP	c, e	Levrero <i>et al.</i> , 1991
human cytomegalovirus IE1	anti.	HCMV	c, e	Wilkinson and Akrigg, 1992
<i>E. coli</i> β -galactosidase	parallel	HCMV	c, e	Wilkinson and Akrigg, 1992
HSV 1 ICP4	parallel	ICP4 pro	c, e	Spessot <i>et al.</i> , 1989
HSV 2 ribonucleotide reductase anti.	anti.	MLP	c, e, multiple polyA sequences	Lamarche <i>et al.</i> , 1990
hepatitis B pre-S2 epitope	parallel		c, e	Levrero <i>et al.</i> , 1991
hepatitis B surface antigen	parallel	MLP	c, e	Davis <i>et al.</i> , 1985
hepatitis B surface antigen	parallel	E1	c, e, v/o	Ballay <i>et al.</i> , 1985
hepatitis B surface antigen	parallel	MLP	c, e, v/o, v/ch	Levrero <i>et al.</i> , 1991
hepatitis B surface antigen	parallel	MLP	c, e, E1A recloned downstream of insert	Levrero <i>et al.</i> , 1991
human globin gene	parallel	E1	c, e	Karlsson <i>et al.</i> , 1986
human globin gene anti.	anti.	none	c, e	Karlsson <i>et al.</i> , 1986
measles hemagglutinin	parallel	MLP	c, e	Alkhatib and Briedis, 1988
measles P/C	parallel	MLP	c, e	Alkhatib <i>et al.</i> , 1988
mouse dihydrofolate reductase	parallel	MLP	c, e, E1B present	Berkner and Sharp, 1984
human parainfluenza type 3 gp	parallel		c, e	Ebata <i>et al.</i> , 1992
polymerized human serum albumin	parallel	E1	c, e, v/o	Ballay <i>et al.</i> , 1985
polyoma middle T antigen	parallel	MLP	c, e	Berkner <i>et al.</i> , 1987
polyoma middle T antigen	parallel	MLP	c, e	Massie <i>et al.</i> , 1986
pseudorabies gp50	parallel	MLP	c, e, v/o, v/r	Eloit <i>et al.</i> , 1990
SV40 T antigen	parallel	none	c, e	Thummel <i>et al.</i> , 1981, 1983
tick-borne encephalitis virus NS1	anti.	HCMV	c, e, v/r	Jacobs <i>et al.</i> , 1992

Inserts into hAd2 or hAd5 MLP

polyoma middle T antigen	parallel	MLP	c, c, replication defective vector	Mansour <i>et al.</i> , 1985
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Inserts into hAd2 or hAd5 E3

bovine coronavirus HA-esterase	parallel	none	c, c	Yoo <i>et al.</i> , 1992
HIV-1 envelope proteins	parallel	none	c, c, v/r	Dewar <i>et al.</i> , 1989
HIV-1 gag antigens	parallel	SV40	c, c, v/p	Prevec <i>et al.</i> , 1991
HSV 1 glycoprotein B	parallel	SV40	c, c	Johnson <i>et al.</i> , 1988
HSV 1 glycoprotein C	parallel	SV40	c, c	Witmer <i>et al.</i> , 1990
HSV 1 glycoprotein E	parallel	SV40	c, c	Hanke <i>et al.</i> , 1990
HSV 1 glycoprotein I	parallel	SV40	c, c	Hanke <i>et al.</i> , 1990
HSV 1 thymidine kinase	anti.	tk pro	c, c	Haj-Ahmad and Graham, 1986
hepatitis B surface antigen	parallel	none	c, c	Morin <i>et al.</i> , 1987
mouse hepatitis coronavirus spike, nucleocapsid, membrane proteins	parallel	SV40	c, c, v/r	Wesseling <i>et al.</i> , 1993
rabies glycoprotein	parallel	SV40	c, c, v/r, v/d	Prevec <i>et al.</i> , 1990
rotavirus VP4	parallel	none	c, c	Gorziglia and Kapikian, 1992
rotavirus VP7sc	parallel	SV40	c, c, v/r	Both <i>et al.</i> , 1993
vesicular stomatitis virus G	parallel	tk pro	c, c, v/r, v/d,	Schneider <i>et al.</i> , 1989
	anti.		v/o	

Inserts into group C Ad between E4 and right ITR

hepatitis B surface antigen	parallel	none	c, c, E3 region deletion	Saito <i>et al.</i> , 1985
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Orientation: parallel to the E1 and E3 regions, or anti. (antiparallel) to the E1 and E3 regions

Promoters: MLP - adenovirus major late promoter SV40 - SV40 early promoter

HCMV - human cytomegalovirus immediate early promoter

tk pro - HSV 1 thymidine kinase promoter

Comments: c - construction, e - expression studies, v/r - vaccination of rodents, v/d - vaccination of dogs, v/p - vaccination of primates, v/o - vaccination, other species

hemagglutinin (HA) expression off MLP in an E1 defective vector at 65-130% of those in measles virus-infected 293 cells, while in cells unable to replicate virus the levels of HA were at 35%. Eloit *et al.* (1990) replaced hAd5 E1A with an MLP/pseudorabies gp50 cassette and observed high levels of expression in cells irrespective of complementation for the E1A mutation. Over an extended period higher expression was obtained in HeLa cells which fail to complement the E1A mutation (Eloit *et al.*, 1990). Levrero *et al.* (1991) report a yield of 6-11 μg of hepatitis B surface antigen per 10^6 cells when expressed off the MLP in parallel to E1. The HCMV major

immediate early (IE) promoter is a second powerful exogenous promoter used for foreign gene expression (Boshart *et al.*, 1985).

The HCMV IE promoter enables constitutive expression of foreign sequences and, in cell culture, can be stimulated *in trans* up to 60 fold by phytohemagglutinin (PHA), forskolin, or other inducers of transcription factors. An approximate yield of 27 μg of β -galactosidase/6 cm diameter cell culture dish when expressed off the HCMV IE promoter in an E1⁻ background has been reported (Wilkinson and Akrigg, 1992).

Expression Of Foreign Inserts In The E3 Region

Expression of inserts in the E3 region of adenovirus results in replicative expression vectors. Inserts in parallel to the E3 promoter are expressed off the E3 promoter early in infection and may be expressed off the major late promoter late in infection. Dewar *et al.* (1989) inserted HIV-1 env gp160 sequences into hAd5 E3 (deletion of sequences from 79.4-84.0 mu) and detected both expression of gp160 and processing to gp120 or gp41 early and late in the viral cycle. A yield of 0.8-1 μg of hepatitis B surface antigen per 10^6 cells was obtained by expression off the E3 promoter alone (Lubeck *et al.*, 1989). The construction of cassettes with exogenously-derived promoters (such as the SV40 early promoter) does not always ensure regulated expression. Johnson *et al.* (1988) failed to detect herpes simplex virus glycoprotein B (gB)-related transcripts originating from the SV40 promoter cassette in E3 and, instead, observed transcriptional initiation at an upstream region (*i.e.* likely from either the E3 or ML promoters) resulting in gB accumulation from 12 to 60 hours p.i.. Synthesis of gB was not detected early (*i.e.* prior to 10 hr p.i.) indicating expression off the MLP commenced once viral DNA replication had initiated. Johnson's group also provided evidence that the sequence downstream of the SV40 TATA box, CCGAGG, acts as a cryptic 3' splice acceptor site (Johnson *et al.*, 1988). Mittal *et al.* (1993) evaluated firefly luciferase expression from the SV40 promoter in the E3 deletion. In the presence of cytosine β -D-arabinofuranoside (araC) - an inhibitor of DNA replication - the researchers were able to detect only 0.4% of luciferase activity (compared to conditions of normal DNA replication) indicating that most expression of luciferase was directed off the major late promoter. Levels of luciferase production were measured as 23 $\mu\text{g}/10^6$ HeLa

cells at 36 hours p.i.. Expression of inserts off the SV40 promoter in cassettes placed anti-parallel to E3 result in very low expression levels indicating the low efficiency of the promoter (Johnson *et al.*, 1988). Conditions for maximal expression and total yields vary and cannot be accurately predicted from past research.

ADENOVIRUS AS A VIRAL VACCINE VECTOR AGAINST RHABDOVIRUSES

Human adenovirus type 5 has been used previously by researchers in our laboratory for the expression of glycoprotein sequences from the rhabdoviruses vesicular stomatitis virus (VSV) and rabies (Prevec *et al.*, 1989, 1990; Schneider, 1989; Schneider *et al.*, 1989; R. Spessot and L. Prevec, personal communication). Schneider and coworkers (Schneider, 1989; Schneider *et al.*, 1989) inserted a cassette of VSV Indiana glycoprotein cDNA sequences under the control of the HSV-1 thymidine kinase promoter in the E3 region *Xba*I deletion in both parallel and antiparallel orientations. Expression of the glycoprotein was detected early in infection, prior to viral DNA replication, and synthesis was not diminished in the presence of AraC. Optimal expression was obtained by placing the cassette in parallel orientation to the E3 promoter, and transcript analysis suggested that promoters upstream of the exogenous promoter were activated. Animal studies indicated that species non-permissive for adenoviral replication were able to elicit a positive immune response to VSV G (Prevec *et al.*, 1989). Calves, piglets, and dogs, administered the vector by subcutaneous or oral routes, developed high titers of neutralizing antibody to VSV while intraperitoneal immunization of mice with the recombinant vector resulted in protection against a challenge with VSV. A related recombinant vector, AdG1, with VSV Indiana G expressed off an exogenous SV40 early promoter also gave favorable expression data in a cell culture system and elicited a good neutralizing response in mice (R. Spessot and L. Prevec, personal communication).

Prevec *et al.* (1990) reported the effectiveness of a recombinant Ad5 vector expressing the rabies ERA strain glycoprotein sequence. In the construct, rabies G cDNA was cloned between SV40 early promoter and polyadenylation sequences; this cassette was placed into the E3 region *Xba*I deletion in parallel orientation to the E3 promoter and the resulting virus designated AdRG1. AdRG1 was given in doses of 10^6 or 10^7 pfu to female Swiss mice by either

the oral or intraperitoneal route; at four weeks post-immunization mice developed high neutralizing antibody titers although seroconversion rates were higher for mice administered AdRG1 intraperitoneally (Prevec *et al.*, 1990). A dose of 10^7 ensured survival of mice (Prevec *et al.*, 1990). Dogs, nonpermissive for human adenovirus replication, developed high titers of antibody by week 4 after either a subcutaneous or intranasal inoculation with 5×10^5 pfu of the recombinant (Prevec *et al.*, 1990). The favorable data obtained with AdRG1 lead to its evaluation as a live virus vaccine vector to be given to wildlife populations. Charlton *et al.* (1992) administered a wide dose range of AdRG1 to striped skunks (presented as a food bait, or given directly into the mouth or intramuscularly) and to red foxes (given directly into the mouth). Most animals developed moderate to high antibody titers between 14 and 30 days post-vaccination. In addition, twelve skunks and fifteen foxes survived a challenge with rabies virus given at 90 or 107 days post-vaccination, respectively (Charlton *et al.*, 1992). The effectiveness of AdRG1 was also demonstrated in raccoons which are considered a significant vector species for rabies in North America (Charlton *et al.*, 1992). Thus, AdRG1 was demonstrated to be a potential rabies vaccine for wildlife populations especially promising for skunks as this species fails to respond to attenuated rabies strains currently employed in wildlife vaccination programs.

RESEARCH UNDERTAKEN

Human adenoviruses have been repeatedly demonstrated to be suitable expression vectors for vaccine use and work in our laboratory has demonstrated the usefulness and practicality of vaccinating animal species with recombinant hAd5 virus so as to offer protection against rabies infection. AdRG1 was demonstrated to be a promising vaccine against rabies in wildlife populations, specifically foxes, skunks, and raccoons. Although these species are nonpermissive for adenovirus replication, sufficient expression of rabies glycoprotein was obtained *in vivo* to obtain a good immune response (Prevec *et al.*, 1990; Charlton *et al.*, 1992). Compared to other recombinant vectors developed in our laboratory, AdRG1 expressed very low levels of rabies glycoprotein in cell culture conditions, requiring infection of permissive cells, such as HeLa, at *moi*'s of greater than 100 pfu/cell for immunodetection of the glycoprotein (R. Spessot, B. Christie, and L. Prevec, personal communication). As levels of foreign protein expression might

directly determine vaccine dose, higher levels of rabies glycoprotein production by recombinant hAd5 might decrease the dose of virus administered in a bait. An additional criterion for vaccines targetting wildlife populations requires the demonstrated stability and effectiveness of the preparation under environmental conditions; *i.e.*, the levels of protein expression should be sufficient to accomodate a predicted decrease in the titer of the recombinant Ad vaccine prior to bait uptake by the target animal. One project was to determine the optimal conditions required for rabies glycoprotein expression by recombinant hAd5. This was carried out by comparing the effect of deletions in E1 and E3 and the effect of exogenous promoter and polyadenylation sequences on rabies G expression in cell culture and *in vivo* animal models.

Studies of the murine immune response to rabies ERA strain glycoprotein have defined several epitopes and domains required for the induction of a neutralizing antibody response and cytotoxic T lymphocyte response. A second project was to determine the contribution of individual domains of rabies G in an immune response by constructing a series of chimaeric proteins between rabies glycoprotein and the related glycoprotein of vesicular stomatitis virus serotype Indiana. In turn, this provided information concerning VSV G epitopes, critical regions affecting the conformation and presentation of both rabies and VSV G epitopes, and may provide a model for the development of chimaeric proteins as "subunit"-type vaccines.

CHAPTER 2

MATERIALS AND METHODS

BACTERIAL CELLS, GROWTH AND STORAGE

Escherichia coli strains LE392 (Murray *et al.*, 1977) and DH5 α (Gibco BRL Life Technologies, Inc., Canada) were grown in Luria-Bertani (LB) broth (10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl per liter of dH₂O; sterilized by autoclaving) or on solid LB agar (15 g bacto-agar per liter of LB prior to autoclaving) at 37°C. Bacterial cultures were stored at 4°C, or as frozen permanents in 20% glycerol at -70°C.

MAMMALIAN CELLS, GROWTH AND STORAGE

Several mammalian cell lines were used in infection studies and expression assays: HeLa (human aneuploid cervical carcinoma), 293 (human embryonic kidney cells transformed with hAd5 DNA; Graham *et al.*, 1977), MDCK (Maden Darby Canine Kidney), MDBK (Maden Darby Bovine Kidney), MRC-5 (human diploid lung fibroblasts), KB cells (human oral epidermoid carcinoma), and L cells (mouse fibroblasts). Cell lines were maintained as monolayers in gamma-irradiated polystyrene cell culture dishes (Falcon® and Nunclon®).

293 cells were maintained in minimal essential medium (MEM) F-11 supplemented with 10% volume/volume (v/v) newborn calf serum (Gibco BRL Life Technologies, Inc.), penicillin-streptomycin (100 U/ml and 100 μ g/ml final concentration, respectively; Gibco BRL Life Technologies, Inc.), and 25 U/ml nystatin suspension (Gibco BRL Life Technologies, Inc.). 293 monolayers were dispersed by incubating in the presence of versene (1.4 M NaCl, 6.8 mM EDTA, 26 mM KCl, 15 mM KH₂PO₄, 15 mM Na₂HPO₄, 0.2% glucose) or 1% (v/v) sodium citrate solution at 37°C. Experiments with 293 cells were restricted to passage numbers not exceeding 50.

MRC-5 cells were maintained in MEM F-11 medium supplemented with 10% (v/v) fetal bovine serum (Bocknek Laboratories, Inc.), penicillin-streptomycin (100 U/ml and 100 µg/ml, respectively), and 25 U/ml nystatin suspension. Monolayers of MRC-5 were dispersed by incubating with 0.5% trypsin (Gibco BRL Life Technologies, Inc.) at 37°C. Other cell types were maintained as monolayers in α-MEM medium supplemented with 10% (v/v) newborn calf serum, penicillin-streptomycin (100 U/ml and 100 µg/ml, respectively), and 25 U/ml nystatin suspension, and were dispersed with 0.5% trypsin. 199-met medium supplemented with 5% (v/v) newborn calf serum and penicillin-streptomycin (100 U/ml and 100 µg/ml, respectively) was used for *in vitro* labelling studies.

Cells to be frozen down as stocks were grown to 80-90% confluency in 150 mm dishes. Cells were washed once with phosphate buffered saline (PBS: 8 g NaCl, 0.2 g KCl, 1.15 g Na₂HPO₄, 0.2 g KH₂PO₄ per liter of dH₂O) and dispersed with trypsin. Cells were resuspended in 4 ml of fetal calf serum containing 10% (v/v) dimethyl sulfoxide and aliquoted into 2 ml Nalgene® cryotubes. Cells were packaged into a styrofoam rack, slowly frozen by storage for several hours at -20°C, then overnight at -70°C before transfer to a liquid nitrogen cell storage tank.

VIRUSES

Wild type human adenovirus (hAd) 5, hAd5 strain *d1309* (containing an E3 deletion which removes both the *EcoRI* site (83.6 mu) and *XbaI* site (84.8 mu) and a base substitution at the 78.3 mu *XbaI* site; Jones and Shenk, 1979), AdlacZ (containing SV40 early promoter, β-galactosidase, and SV40 polyadenylation sequences inserted into the E3 region *XbaI* deletion; B. Christie and L. Prevec, personal communication), AdRG1 (containing SV40 early promoter, rabies (ERA strain) glycoprotein cDNA, and SV40 polyadenylation sequences inserted into the E3 region *XbaI* deletion; Prevec *et al.*, 1990), and AdG1 (containing SV40 early promoter, VSV (Indiana) glycoprotein cDNA sequences inserted into the E3 region *XbaI* deletion; R. Spessot and L. Prevec, personal communication) were utilized in these studies. VSV Indiana HR pp2 virus (L. Prevec, personal communication) was used in VSV neutralization assays.

PLASMIDS

The following plasmids were used in the construction of recombinant vectors. pUC8 and pUC9 are Amp^R vectors containing opposite orientations of a multicloning site (*EcoRI SmaI BamHI SalI PstI HindIII*; Messing, 1983; Yanisch-Perron *et al.*, 1985). pSV2X3 contains the complete SV40 early promoter and polyadenylation sequences separated by a multicloning site (*HindIII BamHI EcoRI SmaI XhoI*) (Prevec *et al.*, 1990). pBRRG, a pBR322 derivative, contains the rabies (ERA strain) glycoprotein cDNA as an 1.65 kb *EcoRI-BamHI* fragment (Connaught Laboratories, Inc., Canada). pSV2X3RG (Prevec *et al.*, 1990) is a pSV2X3 derivative containing the *EcoRI-BamHI* fragment of rabies (ERA strain) glycoprotein cDNA cloned into the unique *SmaI* site and oriented parallel to the SV40 promoter. pSV2VSVG is a pSV2X3 derivative containing the VSV (Indiana strain) glycoprotein cDNA sequences in the multicloning site (P. Lee and L. Prevec, personal communication). pFGdX1 (Haj-Ahmad and Graham, 1986), pFG144K5 (Bett *et al.*, 1993), pAB14 (Bett *et al.*, 1993), and pAB16 (Bett *et al.*, 1993) are pBR322-derived plasmids containing viral sequences from the right end of the Ad5 genome (map units 59.5-100) with deletions in the E3 region and unique restriction enzyme cloning sites. pFGdX1 (Amp^R) and pFG144K5 (Nm^R) contain a deletion of the 1.9 kb *XbaI* D fragment, and utilize the unique remaining *XbaI* site as a cloning site for foreign inserts. pAB14 and pAB16 both carry a 2.5 kb deletion of the *BglII* F and M fragments: pAB14 contains a unique *XbaI* cloning site, whereas pAB16 has a multicloning site (*XbaI BamHI XhoI SalI ClaI*). pKDAdMLP-5a contains the Ad2 major late promoter, tripartite leader cDNA, and 5' and 3' splice sites as a 0.75 kb *SacII-BamHI* fragment (Berkner and Sharp, 1984, 1985; obtained from J.A. Hassell, McMaster University). pHCMVsp13 is a pBR322 derivative containing left-end sequences of Ad5 (nt 1-5,788) with an *AflIII/SspI* E1 region deletion (nt 342-3,533). The deletion was replaced with the *SpI* binding site for protein IX, the human cytomegalovirus (HCMV) immediate early (IE) promoter, and a polylinker region (A. Bett and F.L. Graham, personal communication). pFG173, derived from pFG140 (Graham, 1984), contains the entire Ad5 genome and a lethal deletion in the E3 region (F.L. Graham, personal communication). pJM17, a derivative of pFG140, contains the entire Ad5 genome with a 4.4 kb DNA segment inserted into the E1 region at 3.7 mu and exceeds the packaging capacity of the adenovirus capsid (McGrory *et al.*, 1988). Plasmids constructed as

part of this project are described in subsequent chapters.

OLIGONUCLEOTIDES

Oligonucleotides (Table 2.1) were synthesized by The Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University. One OD of oligonucleotide was resuspended in 100 μ l of sterile water to give a concentration of 0.32 μ g/ μ l.

TABLE 2.1: OLIGONUCLEOTIDE SEQUENCES

Linker	Sequence	Use
AB698	5' CAAAATGCAATACTGCAAG 3'	RV1 construction
AB699	5' GCAGTATTGCATTTTGCATG 3'	RV1 construction
AB764	5' AAAGGGAGTGAGACTTG 3'	rabies G cDNA nucl. 661-687
AB765	5' CGGAGAGCTATCATCCC 3'	VSV G cDNA nucl. 624-640
AB948	5' CTAGTTGAGCGGGACAG 3'	hAd5 E3 region 5' to <i>Bgl</i> III deletion
AB973	5' GCCAGATTCGCATG 3'	VR1 construction
AB974	5' CGAATCTGGCTGCA 3'	VR1 construction
AB975	5' GCCAGATTCCTGAATGCCAAGAAGCATG 3'	VR2 construction
AB976	5' CTTCTGGGCATTCAGGGAATCTGGCTGCA 3'	VR2 construction
AB1167	5' GGCAAGGAGGTGCTGCTG 3'	hAd5 E3 region 3' to <i>Bgl</i> III deletion, opposite strand
AB1180	5' GTCTGGTATCGTGTA AAA 3'	rabies G cDNA opposite strand corresponding to nucl. 68-84
AB1181	5' ACTATGTTGAACTTGCA 3'	VSV G opposite strand, nucl. 46-62
AB1185	5' CATGTCTAGA 3'	<i>Xba</i> I linker

ANTIBODIES

Rabies glycoprotein was detected with 10 μ l of rabbit antiserum (J. Campbell, University of Toronto, Canada), or with 3 μ l of ascites-derived monoclonal antibodies 10AA9-2A6, 10EC9-1D5, or 10ED8-2B8 (A. Wandeler, Animal Disease Research Institute, Nepean, Canada). VSV glycoprotein was precipitated with 10 μ l of rabbit anti-VSV Indiana polyserum (Bell *et al.*, 1984) or rabbit anti-VSV Indiana glycoprotein polyserum (L. Prevec). Mouse monoclonal antibody H2-19 was specific for Ad5 72K protein (5 μ l per reaction; Rowe *et al.*, 1984). A rabbit polyclonal serum was used to detect Ad5 proteins (10 μ l per reaction; G. Martins and L. Prevec). Ad5 hexon protein was detected with monoclonal antibody 9F6-6 (2 μ l per reaction; J. Williams, University of Pittsburgh, USA), while Ad5 protein IX was detected with a monoclonal raised against Ad2 protein IX (2 μ l per reaction; W.C. Russell, University of St. Andrews, Scotland). Other monoclonal antibodies directed against Ad5 are characterized in APPENDIX 2.

ENZYMES

Enzymes for restricting or modifying DNA were purchased from one of several suppliers: Gibco BRL Life Technologies, Inc., New England Biolabs (NEB) Ltd., Boehringer Mannheim Canada, Ltd., and Pharmacia Canada Inc.. Enzymes, supplied at concentrations of 10-12 U/ μ l in 50% glycerol, were stored at -20°C unless otherwise suggested by the supplier. 5X or 10X buffers recommended by the manufacturers for optimal reaction conditions were also provided.

RECOMBINANT DNA TECHNIQUES

Restriction Of Plasmid DNA

To prepare DNA for electrophoretic analysis or for further cloning reactions, plasmid DNA was digested with restriction enzymes. Type II restriction enzymes recognize palindromic sequences, usually four to six nucleotides in length, and cleave at specific sequences within or

adjacent to the recognition sequence to generate fragments with blunt ends (when cleavage occurs at the axis of dyad symmetry) or with protruding single-stranded termini. Typical conditions for restriction reactions consisted of 0.5-1.0 μg of purified plasmid DNA incubated with 2-5 units of restriction enzyme in a 1X buffer with a total reaction volume of 20-30 μl . Reactions were incubated for several hours at 37°C unless otherwise indicated. Manufacturers supplied concentrated buffers containing appropriate salt concentrations for optimal enzyme activity.

For multiple enzyme digests buffers were selected that would enable at least 50% of the optimal activity for each restriction enzyme where enzyme activity was defined as the number of units of enzyme required to cleave 1 μg of lambda DNA to completion in one hour. As restriction enzyme activity was potentially inhibited at final glycerol concentrations exceeding 12% (v/v), reaction volumes were adjusted appropriately. If a single compatible buffer was not obtained, DNA was digested first with the enzyme that worked in the buffer of lower ionic strength, followed by an adjustment in the salt concentration and digestion with the second restriction enzyme. Alternatively, the DNA was precipitated with 2-3 volumes of 95% ethanol, dried, and dissolved in sterile water before proceeding with the second restriction reaction. Partial DNA digests were carried out by diluting the restriction enzymes to 1 unit per 3-5 μg of plasmid DNA and allowing the reaction to proceed for 5-15 minutes.

Removal Of 5' Phosphate Groups

Linearized plasmid vector DNA was treated with calf intestinal alkaline phosphatase (CIAP) to remove 5' phosphate groups preventing vector recircularization. Reaction conditions consisted of 10 μg of restricted plasmid DNA and 2 u of CIAP enzyme in CIAP buffer (50 mM Tris, pH 8.0, 1 mM MgCl_2) for one hour at 37°C. Plasmid DNA was precipitated with three volumes of 95% ethanol and washed with 70% ethanol.

Blunting Of Linearized DNA Ends

3' recessed ends of linearized plasmid DNA were filled using the large fragment of DNA polymerase I (known as Klenow fragment) derived from *E. coli*. Klenow enzyme lacks the 5'-3'

exodeoxyribonuclease activity of DNA polymerase I but contains the 3'-5' exodeoxyribonuclease activity. Reaction conditions consisted of 10 μg of DNA, 10 mM dNTP mix (containing 2.5 mM each of dATP, dGTP, dCTP, and dTTP), NT buffer (50 mM Tris, pH 7.2, 10 mM MgSO_4 , 0.1 mM DTT, and 50 $\mu\text{g}/\text{ml}$ of bovine serum albumin), and 2 μl of Klenow enzyme for 30 min at RT. The enzyme was inactivated for 15 min at 65°C, and the DNA was precipitated with ethanol and washed.

Phosphorylation Of DNA

T4 polynucleotide kinase was used to transfer the γ -phosphate group from ATP to 5' hydroxyl termini of DNA. Linker oligonucleotide was resuspended in 100 μl of sterile water per OD unit to give an approximate concentration of 500 ng/ μl . Up to 5 μg of DNA was phosphorylated in a reaction containing 1 mM ATP and 10 U of T4 polynucleotide kinase in kinase buffer (50 mM Tris, pH 7.6, 10 mM MgCl_2 , 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA) for 30 min at 37°C. The enzyme was inactivated for 10 min at 65°C.

Electrophoresis Of DNA Fragments

Gels for separating DNA fragments were prepared in horizontal electrophoresis tanks. Tris-acetate (TAE) buffer was prepared as a 50X stock (2M Tris-acetate, pH 7.9, 0.25 M sodium acetate, 0.05 EDTA) and diluted to 1X for use as both the gel and tank buffer. Agarose (0.9% weight per volume (w/v)) was dissolved in 1X TAE by boiling. This concentration of agarose offered a 0.5 - 7.0 kb fragment separation range. Electrophoresis was carried out with the gel submerged in 1X TAE containing 0.165 $\mu\text{g}/\text{ml}$ of ethidium bromide. To determine and confirm the sizes of DNA fragments marker DNA consisting of either 1 $\mu\text{g}/10 \mu\text{l}$ of lambda DNA digested with *Bsr*EII or 1 kb marker (Gibco BRL Life Technologies, Inc.) was included on the gel alongside the samples. Gels were run at less than 4 V/cm. To view the DNA fragments, the gel was transferred to an ultraviolet illuminator box, and photographed with a Polaroid camera using Polaroid type 57 film.

Purification Of DNA Fragments From Agarose Gels

Purification of specific DNA fragments from agarose gels followed the Bio 101 Inc.[®] Gene Clean[®] protocol. The fragment was excised with a sterile scalpel blade, crushed by passage through a syringe, and transferred to a 1.5 ml microfuge tube. Sodium iodide solution (2.5 volumes of 6 M NaI, 0.12 M Na₂SO₃) was added to the tube, and the agarose dissolved by heating in a 50°C waterbath for 5 min. Glass milk (20 µl per ml of NaI/agarose/DNA solution; Bio 101 Inc.[®]) was added to the tube, and tubes were kept on ice for 5 min. Tubes were briefly centrifuged and the supernatant was removed. The DNA/glass milk was washed three times with 700 µl of cold New Wash solution (42% ethanol, 10 mM Tris, pH 7.4, 0.5 mM EDTA, 50 mM NaCl) to remove any residual sodium iodide: tubes were gently tapped to resuspend the DNA/glass milk pellet and then recentrifuged. After the final wash, traces of New Wash solution were carefully pipetted off. DNA was eluted from the glass beads by adding 20 µl of sterile water to the tubes, vortexing, heating at 50°C for 5 min, centrifuging, and recovering the supernatant containing the DNA fragments.

Ligations

Bacteriophage T4 DNA ligase was used to join DNA by forming phosphodiester bonds between phosphate residues at the 5' termini of DNA and adjacent 3'-hydroxyl groups. Ligation reactions contained 0.5-1.0 µg of dephosphorylated vector DNA, a 10-fold molar excess of the insert sequence, 2 U of T4 DNA ligase, 1 mM ATP, and ligase buffer (50 mM Tris, pH 7.7, 10 mM MgCl₂, 20 mM dithiothreitol). The total volume of the reaction did not exceed 20 µl. Blunt-end reactions were carried out at 14°C overnight whereas DNA fragments with recessed ends were ligated for several hours at RT. Reactions were inactivated at 65°C for 5 min prior to transformation or electroporation.

Transformation Of *E. coli*

E. coli strains DH5 α or LE392 were grown as 10 ml overnight cultures in LB broth at 37°C. One ml of culture was inoculated into 100 ml of LB and grown in an incubator shaker at 37°C until OD₅₅₀ was 0.5-0.6. Bacterial cultures were chilled on ice for 10 min and centrifuged for 5 min at low speed at 4°C. The medium was discarded and cells were resuspended in 50 ml of ice-cold transformation buffer (50 mM CaCl₂, 10 mM Tris, pH 8.0) and left on ice for 15 min. After a second centrifugation (4000 rpm, 4°C, for 5 min) cells were resuspended in 3 ml of transformation buffer, and stored at 4°C overnight prior to transformation.

Per transformation, 0.1-1 μ g of plasmid DNA or 3-5 μ l of a ligation mix was added to 100 μ l of chilled, competent cells and stored on ice for 30-60 min. Cells were heat-shocked at 42°C for two min, 1 ml of LB was added to each tube, and cells were allowed to recover by incubating at 37°C for one hour. Dilutions of cells (10⁻¹-10⁻³) were plated on LB agar supplemented with 25-50 μ g/ml of the appropriate antibiotic and incubated overnight at 37°C.

Electroporation Of *E. coli*

To prepare bacterial cells for electroporation, 10 ml of a fresh overnight culture of DH5 α was inoculated into a litre of LB broth. Cells were grown in an incubator shaker at 37°C until the OD₆₀₀ was between 0.5 and 0.8. The cells were transferred to centrifuge bottles, chilled on ice for 30 min, and pelleted at 4000 rpm for 10 min at 4°C. To minimize warming conditions the remaining steps were carried out on ice. Medium was decanted and cells were resuspended in a litre of ice-cold sterile dH₂O. The cells were centrifuged as previously, resuspended in 500 ml of ice-cold dH₂O, centrifuged, resuspended in 20 ml of sterile 10% glycerol, centrifuged, and finally resuspended in 3 ml of sterile 10% glycerol. Cells were aliquoted in 50 μ l volumes into pre-chilled 0.5 ml microfuge tubes, and flash-frozen in an isopropanol-dry ice bath. Cells were stored at -70°C.

To prepare for electroporation, one vial of bacteria per DNA sample was thawed on ice. One μ l of ligation reaction was added to the cells, gently mixed, and transferred to pre-chilled electroporation cuvettes. The Bio Rad Gene Pulser[®] unit was preset to a capacitance of 25 μ F, a voltage of 2.25 kV, and a resistance of 200 Ω . The cuvette was placed into the safety chamber

and pulsed for approximately 5 seconds. After pulsing, 1 ml of SOC media (2% bacto-tryptone, 0.5% bacto-yeast extract, 10 mM NaCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose, 2.5 mM KCl; sterilized by autoclaving) was added to the cuvettes, the mixture was quickly pipetted several times, and cells were transferred to inoculum tubes. The cell suspension was left on ice for 10 min, then transferred to an incubator shaker for 1 hr (Amp^R plasmids) or 2 hrs (Nm^R plasmids). Dilutions of 10⁻¹, 10⁻², and 10⁻³ were plated on LB agar containing the required antibiotic and antibiotic-resistant colonies were grown overnight at 37°C.

Large-Scale Plasmid Preparation

Both methods for plasmid preparation are based on alkali lysis of bacterial cells and are adaptations of those described in Sambrook *et al.* (1989). For large-scale plasmid preparation bacterial colonies containing the desired plasmid were inoculated into 10 ml of LB supplemented with 50 µg/ml of appropriate antibiotic and grown as overnight cultures at 37°C. One ml of culture was inoculated into a litre of LB supplemented with antibiotic and grown in an incubator-shaker overnight at 37°C. To increase plasmid yield, cultures were grown in Terrific Broth (TB) (12% (w/v) bacto-tryptone, 24% (w/v) bacto-yeast extract, 0.4% (v/v) glycerol; sterilized by autoclaving) supplemented with 10% (v/v) potassium phosphate solution (0.17 M KH₂PO₄, 0.72 M K₂HPO₄). Cultures were transferred to one litre centrifuge bottles and pelleted at low speed for 10 min. The pellet was resuspended in 40 ml of lysozyme buffer (50 mM glucose, 25 mM Tris, pH 8.0, 10 mM EDTA, pH 8.0, 5.0 mg/ml lysozyme) and incubated for 15 min at RT. Eighty ml of alkaline cell lysis solution (1% sodium dodecyl sulfate, 0.2 N NaOH, prepared fresh) was added and the mixture was incubated on ice for 5 min. Chromosomal DNA, high MW RNA, and membrane complexes were precipitated by the addition of 40 ml of 5 M potassium acetate solution, pH 4.8, and incubation on ice for 30 min. Distilled water (10 ml) was swirled into the mix, and cellular debris was pelleted at low speed for 10 min. The supernatant was filtered through several layers of cheesecloth into 500 ml centrifuge bottles. DNA was precipitated by adding 100 ml of isopropanol, incubating at RT for 20 min, and pelleting at low speed for 10 min. The supernatant was decanted and the bottle left inverted for 15 min. The DNA pellet was redissolved in 5 ml of 0.1X SSC (15 mM NaCl, 1.5 mM

Na• citrate, pH 7.0) and remaining protein degraded by the addition of 2 ml of pronase® solution (1 mg/ml pronase® in 10 mM Tris, pH 7.8, 10 mM EDTA, 0.5% SDS) and incubation at 37°C for 30 min. *ultraPURE*® caesium chloride (8.4 g; Gibco BRL Life Technologies, Inc.) was dissolved in the DNA solution, the tubes were stored on ice for 30 min, and degraded protein pelleted by centrifugation at 3000 rpm for 10 min. The plasmid-containing supernatant was transferred to 16 x 76 mm Quick-Seal® centrifuge tubes (Beckman Instruments, Inc.). Ethidium bromide (0.4 ml of a 10 mg/ml stock) followed by light paraffin oil were added to the tubes. Plasmid was banded by equilibrium centrifugation at 55,000 rpm, 15°C for 16 hours. Two bands of DNA intercalated with ethidium bromide are obtained in the center of the gradient. The lower band, containing closed circular plasmid DNA, was removed with an 18 gauge needle leaving a fainter upper band, containing linear bacterial DNA and nicked circular plasmid DNA. Ethidium bromide was removed from the DNA solution by extracting three times with an equal volume of caesium chloride (CsCl)-saturated 1-propanol. The CsCl was removed from the solution by diluting with two volumes of 0.1X SSC or water, and precipitating with two volumes of ice-cold 95% ethanol followed by centrifugation at 3000 rpm for 10 min. The DNA pellet was washed with 70% ethanol, dried, and dissolved in 0.3 - 1 ml of 0.1X SSC. The optical density at 260 nm was measured and used to calculate the concentration of the plasmid DNA.

Small-Scale Plasmid Preparation

Colonies obtained after transformation were grown as overnight cultures at 37°C in 3 ml of LB broth supplemented with 50 µg/ml of the appropriate antibiotic. One ml of the cultures was transferred to 1.5 ml microfuge tubes, centrifuged for one minute, and the media was aspirated. Pellets were resuspended in 100 µl of lysozyme solution (as for large-scale preparations) and left at RT for 10 min. 200 µl of alkaline SDS was added to each tube and tubes were gently swirled and left on ice for 5 min. Cellular debris was precipitated by adding 150 µl of 5 M potassium acetate (pH 4.8), vortexing, and incubating on ice for 20 min. Samples were centrifuged 5 min and supernatants transferred to new microfuge tubes. Plasmid was precipitated with 1 ml of isopropanol; tubes were incubated for 10 min at RT and centrifuged for 10 min. Pellets were washed first with 100 µl of (0.1 M sodium acetate, 50 mM Tris, pH 8.0)

and 200 μ l of cold isopropanol, followed by a wash with 70% ethanol. Pellets were dried and plasmid DNA resuspended in 100 μ l of sterile water of which 10-15 μ l was used per restriction digest.

Sequencing Of Plasmid DNA

The same stock of 1X TBE was used in both the sequencing gel and the running well buffers. 1X TBE, made up as 24.22 g Tris, 10.27 g boric acid, and 0.75 g EDTA dissolved in two liters of distilled water, provided a sufficient volume for the preparation of one sequencing gel. The 7% acrylamide/urea gel solution (6.65% (w/v) acrylamide, 0.35% (w/v) bisacrylamide, 42% (w/v) urea in 100 ml 1X TBE) was dissolved at 65°C, cooled to room temperature, filtered through a 0.45 μ m filter unit, and chilled on ice. Glass plates (37.5 cm x 42.5 cm, 37.5 x 45 cm) were washed with glass detergent, and thoroughly rinsed with distilled water, followed by a rinse with 95% ethanol. One side of the longer plate was evenly treated with 5 ml of Sigmacote®, a silicone solution, allowed to dry for five minutes, polished with Kimwipes, and rinsed with distilled water and ethanol. Plates were assembled with 0.4 mm spacers and were bound together on the sides and the bottom with 3M® yellow electrical tape. Immediately prior to casting, 1 ml of 10% (w/v) ammonium persulfate was quickly swirled into the gel solution followed by 40 μ l of TEMED (*N,N,N',N'*-tetramethylethylenediamine) solution. While maintaining the gel at approximately 45° to the lab bench, the gel solution was quickly poured down the side of one of the spacers using a 10 ml syringe as a funnel. Air bubbles were quickly tapped out of the gel or removed with a long 0.2 mm thick plastic strip. The gel was allowed to polymerize for one hour before use, and was ordinarily prepared one day prior to use. After polymerization, the wells were carefully washed with buffer to remove urea and acrylamide, and the gel was pre-run for 30-60 min at 1800 volts.

Sequencing reactions were based on the chain-termination method (Sanger *et al.*, 1977): the synthesis of a DNA strand by a DNA polymerase *in vitro*, and the termination of synthesis as a result of the incorporation of a nucleotide analog that does not permit DNA elongation. The nucleotide analogs consist of 2',3'-dideoxynucleoside 5'-triphosphates (ddNTP) which lack the

3'-OH group necessary for DNA chain elongation. Sequenase[®] (United States Biochemical Corporation) is a modified bacteriophage T7 DNA polymerase featuring high processivity, low 3' to 5' exonuclease activity, and a high efficiency of nucleotide incorporation. The enzyme was acquired as part of a kit containing all required reagents for multiple sequencing reactions.

All sequencing reactions were carried out in 1.5 ml microfuge tubes. Plasmid DNA to be sequenced was alkaline-denatured prior to use: 2-3 μg of plasmid DNA was made up to 18 μl with sterile water and denatured for at least two min at RT in 0.2 M NaOH, then neutralized with 0.4 volumes of 5M ammonium acetate. The DNA was precipitated with 3 volumes of 95% ethanol at -70°C for 10 min, pelleted, washed with 70% ethanol, and vacuum dried. In the annealing reaction, plasmid DNA was resuspended in 7 μl of sterile water, 2 μl of 5X sequencing buffer (200 mM Tris, pH 7.5, 100 mM MgCl_2 , 250 mM NaCl) and 1 μl of primer (20-50 ng) were added to the DNA, and the tubes heated for 2 min at 65°C . The tubes were left in a beaker of 65°C water, and allowed to cool slowly to 30°C . While the tubes were cooling, the remaining solutions were dispensed. The labelling mix (7.5 μM dGTP, 7.5 μM dCTP, and 7.5 μM dTTP) was diluted 1:5 in distilled water. Sufficient Sequenase[®] enzyme for all labelling reactions was diluted 1:8 in cold TE (10 mM Tris, pH 7.5, 1 mM EDTA) and kept on ice. 2.5 μl of each termination mix (ddGTP, ddATP, ddCTP, or ddTTP) was dispensed into labelled eppendorf tubes and pre-warmed to 37°C for at least one minute; each termination mix contained 8 μM of one ddNTP (eg. ddGTP) and contained 80 μM of each dNTP.

For the labelling reaction, 1 μl of 0.1 M DTT, 2 μl of diluted labelling mix, and 0.5 - 1.0 μl of [α -³⁵S]dATP or [α -³²P]dATP (250 $\mu\text{Ci}/25 \mu\text{l}$; Amersham Canada Ltd.) were added to 10 μl of the DNA-primer mixture. The reaction commenced with the addition of 2 μl of diluted Sequenase[®] and was allowed to proceed for 5 min at RT. The labelling step allows primer extension using limiting concentrations of the dNTP and incorporated most of the labelled nucleotide. On completion of the labelling reaction, 3.5 μl was transferred into each of the four labelled termination tubes, and incubation of these tubes was maintained for an additional 5 min at 37°C . During the termination reaction DNA synthesis continues until all growing chains are terminated by dideoxynucleotide incorporation. The reactions were terminated with the addition of 4 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). The tubes were heated to 80°C for 2 min prior to loading 2-3 μl of each

sample per well. Gels were run for several hours at 2100 volts. Upon completion of the electrophoresis, the gel was transferred to Whatman® chromatography paper for support and dried using a Bio-Rad® model 583 gel dryer. The gel was exposed to Kodak X-omat® AR film overnight.

The described reaction enabled sequence determination at a distance of 20 to 300 bases from the primer sequence. To determine the sequence beyond 350 bases several modifications were carried out: the labeling mix was diluted 1:2 to provide a higher concentration of available dNTP, and the labelling reaction was extended to 8-10 min.

RECOMBINANT ADENOVIRUS PREPARATION

Rescue Of Recombinant Virus

Plasmid DNA cotransfections to rescue cloned viral sequences into full length viral genomes were carried out according to the protocol developed by Graham and van der Eb (1973). Cotransfections were carried out with 293 cells as these cells are good recipients of DNA during gene transfer procedures and generate adenovirus plaques rapidly (within 4-6 days). Large 150 mm dishes of 293 cells were washed with 1X citrate-saline, and set up into 60 mm dishes for 70-80% confluency at the time of use, usually the following day. Five dishes were prepared per DNA concentration per plasmid. For the cotransfections, sufficient 1X HEPES buffered saline (20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄·2H₂O, 6 mM glucose, final pH 7.1, autoclaved and stored at 4°C) was prepared with 10 µg/ml salmon sperm DNA (stock 1 mg/ml in water), and vortexed for one minute to fragment the DNA. The volume was calculated so as to use 0.5 ml of HEPES buffered saline/carrier DNA/ 60 mm dish. Plasmid DNA was added to the tubes in a 1:1 proportion with either 5 µg/0.5 ml or 10 µg/0.5 ml of each plasmid used for the cotransfection, and mixed well. 2.5 M CaCl₂ was added to a final concentration of 125 mM (50 µl/ml), the tubes were inverted several times and incubated at room temperature for 15 to 30 minutes until a fine precipitate was observed. The mixture was added dropwise thoroughly over the cells: 0.5 ml of mix per 60 mm dish containing 5 ml of medium. Cells were incubated in a 5% CO₂ in air (90% humidity)

incubator for 4.5 hours at 37°C, medium was then replaced with 10 ml of overlay of 0.5% agarose and F11 supplemented with 5% horse serum, penicillin-streptomycin (100 U/ml and 100 µg/ml final concentration, respectively), 25 U/ml nystatin suspension and 0.2% yeast extract (prepared as 5% stock and autoclaved). Incubation was continued at 37°C until plaques were visible after 5-7 days.

Potential recombinant virus plaque isolates were plaque purified at least twice using 293 cells. For plaque screening 293 cells were split 1:8 into 60 mm dishes to obtain 80-90% confluency at time of infection the following day. Well-isolated plaques were picked by punching out agar plugs with a sterile pasteur pipette, and transferring the agar plug into 1 ml of PBS⁺⁺ (PBS containing 0.01% CaCl₂ and 0.01% MgCl₂). Medium was removed from the 293 cells and replaced with 0.2 ml of virus. Adsorption was allowed to occur at room temperature for 30-60 min, and media was added back. CPE (cytopathic effect, as indicated by the rounding of cells and cell detachment from the substrate) was initially observed after 1-2 days, and was complete after 3-4 days. For viral harvests, dishes were left undisturbed in a laminar biological safety cabinet for 30 min, and medium was carefully removed with a pipette, storing 4 ml of supernatant with 0.5 ml sterile glycerol at -70°C. Residual medium was removed by suction. The remaining loose cells in the dish were treated with 0.5 ml of digestion buffer (1 mg/ml of Pronase® stock in 0.01 M Tris, pH 7.4, 0.01 M EDTA, and 1% SDS), and digested at 37°C for several hours. The viscous lysate was transferred to a microfuge tube, phenol extracted once, and precipitated with 1 ml of 96% ethanol. The DNA was washed several times with 96% ethanol, dried, redissolved in 50 µl of 0.1X SSC, of which 5 µl was analyzed by restriction digestion with *Hind*III. Recombinants corresponding to those required were then plaque purified once more and reanalyzed.

Adenovirus Titration

Adenovirus titres were determined by assaying for viral plaques on 293 cells. Cells were set up in 60 mm dishes one day prior to use so as to have the cells at about 90% confluency at time of infection. Generally, 8 x 60 mm dishes of 293 cells were seeded from each 150 mm dish. Virus dilutions (1:10 series) were prepared in PBS⁺⁺. Medium was removed, cells washed

once with PBS⁺⁺, and 0.2 ml of virus dilution was added per dish. Dishes were tilted to evenly spread the virus, and adsorption was carried out for 30-60 min in a 37°C incubator. Overlay, 10 ml per dish, consisted of a 1:1 ratio of 1% (w/v) agarose in water (sterilized by autoclaving) and 2X MEM F-11 (supplemented with 8 ml of penicillin/streptomycin solution and 25 U/ml nystatin suspension). On 293 cells plaques were usually visible after 5-7 days and were visually counted.

Purification Of Adenovirus

Large scale growth of adenovirus was carried out in spinner cultures of KB cells in 2 litre flasks. Cells were grown at a density of 2×10^5 cells/ml in a 2 litre volume of Joklik's medium supplemented as described previously. Cells were spun down in centrifuge bottles at low speed for 5 min, and medium was poured off retaining 20 ml for cell resuspension and infection with virus at an MOI of 1 pfu/cell. Virus was adsorbed for one hour at 37°C in a CO₂ incubator, and infected cells were transferred back to conditioned medium in 2 litre flasks. Cells were monitored daily for CPE, and harvested 3-4 days post-infection. Infected cells were spun down at low speed at 4°C for 10 min, and resuspended in 4-5 ml of cold 0.1M Tris, pH 8.0, per litre of cell suspension. 5% sodium deoxycholate was added to 1/10th of the cell volume, the mixture was swirled, and allowed to sit on ice for 30 min during which time an increase in viscosity was observed. The volume was accurately measured and recorded. Cellular DNA was sheared using a Tissuemizer[®]: 5 ml aliquots, kept on ice, were processed, the processing consisted of three 15 sec bursts followed by 15 sec coolings. Saturated CsCl stock (in 0.01 M Tris, pH 8.0, 0.001 M EDTA) was added to the cell suspension at a ratio of 1.8 ml per 3.1 ml of virus suspension. The virus was distributed into 50Ti Quickseal[®] (Beckman, Inc.) centrifuge tubes, sealed, and centrifuged in a chilled Beckman 50Ti rotor overnight at 35,000 rpm. The tubes were removed from the rotor, and the viral bands collected from the tube bottoms. Viral bands were pooled into SW50.1 open-top tubes, CsCl-saturated Tris was added to fill the tube, and the virus was centrifuged in a SW50.1 rotor at 35,000 rpm overnight at 4°C. The virus was collected from the bottom of the tube in a small volume into a microfuge tube, and made to 1.5 ml with 10 mM Tris, pH 8.0, following which it was dialyzed overnight at 4°C against two changes of approx.

100 volumes of 10 mM Tris, pH 8.0, 10% glycerol.

Isolation Of Adenoviral DNA

Pronase® stock was prepared as 5 mg/ml in 0.01 M Tris, pH 7.5, and incubated for 15 min at 56°C followed by an hour at 37°C. One volume of digestion buffer (described earlier) was added to a petri dish followed by the dropwise addition of one volume of dialyzed virus stock. The mixture was incubated for a minimum of two hours at 37°C, and was usually allowed to digest overnight. The DNA was phenol-extracted once with TE-saturated phenol, chloroform-extracted, and ethanol precipitated. The DNA was washed several times with 95% ethanol, allowed to dry for one hour at 37°C, and redissolved in 0.1X SSC. Viral DNA was stored at 4°C.

Infected cells in monolayer were washed with PBS prior to the addition of 2 ml (for 150 mm dishes) or 0.5 ml (for 60 mm dishes) of digestion buffer. The digestion buffer was evenly spread across the cells and incubation proceeded overnight at 37°C. The cell lysate was scraped into polypropylene tubes with a silicone or rubber scraper. One volume of saturated phenol was added per volume of lysate, vortexed for 5 min, and centrifuged at maximal microfuge speed at 4°C for 15 min. This was followed by a chloroform extraction, and DNA was precipitated with 1/10 volume of 3 M sodium acetate and 2 volumes of cold 95% ethanol. DNA was centrifuged, washed with 95% ethanol, dried, and redissolved in 0.1X SSC.

PROTEIN EXPRESSION AND DETECTION

³⁵S Labelling Of Viral And Cellular Proteins

To obtain a stock of labelled Ad5 proteins, 150 mm dishes of HeLa cells (75-85% confluency) were washed once with PBS⁺⁺, and infected with Ad5 at an moi of 20 pfu/cell in 2 ml of PBS⁺⁺. Virus was adsorbed for one hour at 37°C, and conditioned medium was added back to the cells. At 20 hours p.i. medium was replaced with 199-met supplemented as described

previously. Cells were labelled at 21 hours p.i. for two hours with 250 μCi of Tran ^{35}S -label (ICN Biomedicals, Inc.) or L- ^{35}S -methionine (Amersham Canada Ltd.). The radioactive medium was carefully removed and the monolayer was washed twice with PBS. The infected cells were solubilized with 8 ml of cold RIPA buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100; filter-sterilized). The cells were incubated at 4°C for 30 min, then harvested using silicone or rubber scrapers. The DNA was sheared by passing the extract four times through a 22G needle, the debris was spun down, and the labelled protein extract used for immunoprecipitations.

For time course studies of viral protein expression, cells were set up in 60 mm dishes to obtain 80-90% confluency for infection. At the time of infection cells were washed once with PBS⁺⁺, counted, and infected with 1-20 pfu of virus per cell (or other MOI's as required). Virus was adsorbed for one hour at 37°C, and conditioned medium was added back to the cells. To examine protein expression under non-replicating conditions, a 1:20 dilution of cytosine arabinofuranoside (araC) (2 mg/ml in sterile dH₂O) was added per dish at the time of infection, 1 hr p.i., and every subsequent 7 hrs. Prior to labelling of cells, medium was replaced with supplemented 199-met medium for 30 min. For the labelling step, cold 199-met was removed from the cells and replaced with 2 ml of 199-met medium containing Tran ^{35}S label (50 μCi per dish) for two hours. After labelling, radioactive medium was carefully removed, cells were harvested into 1 ml of RIPA buffer, incubated on ice for 20 min, and extract passed through a 22G needle to shear the DNA before proceeding with the immunoprecipitations.

To inhibit glycosylation with tunicamycin, infected cell medium was replaced with 199-met containing 15 μg tunicamycin/ml of medium at one hour before metabolic labelling. Pulse labelling with ^{35}S methionine was carried out for 20 min in the presence of tunicamycin at the above concentration. A chase with α -MEM containing 15 μg tunicamycin/ml medium was carried out for 60 min. Cells were harvested as described above.

Immunoprecipitations

In general, for all immunoprecipitations, 300 - 500 μl of labelled cell extract was rotated with 10 μl of a polyclonal antibody, 2-5 μl of a monoclonal antibody produced in ascites fluid,

or 50 μ l of monoclonal antibody in cell culture supernatant. After one or more hours of incubation at 4°C 75-100 μ l of protein A-Sepharose® CL4-B (0.5 g of protein A-Sepharose® in 5 ml RIPA buffer; Pharmacia Ltd.) was added, and the incubation continued overnight at 4°C. Immunocomplexed beads were washed 3 - 5 times with cold RIPA buffer and resuspended in 50 μ l of 2X sample buffer (125 mM Tris, pH 7.2, 5% SDS, 12.5% β -mercaptoethanol, 25% glycerol, bromophenol blue). Samples were stored at -70°C.

For denaturing immunoprecipitations, 500 μ l of protein extract was made 1% with respect to SDS, denatured by heating at 100°C for two min, then placed on ice. Samples were diluted with cold Triton buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1 mM EDTA, 2% Triton X-100; filter-sterilized) to reduce SDS to 1%, then rotated at 4°C overnight in the presence of antibody. 100 μ l of protein A-Sepharose® was added, and incubation continued for an additional 4 hours. Immunocomplexed beads were washed and further processed as described above.

Endoglycosidase H Treatment Of Glycoprotein

Endoglycosidase H (Endo H) cleaves high mannose structures on glycosylated proteins that have not been transported past the medial Golgi. In preparation for an Endo H reaction cell extracts were immunoprecipitated with the required antibody on protein A-Sepharose® as described above. The immunoprecipitated proteins were washed three times with RIPA buffer and denatured in 50 μ l of 1X denaturation buffer (0.5% SDS, 1% β -mercaptoethanol) at 100°C for 10 min. The sample was divided into two microfuge tubes and 3 μ l of 1X digestion buffer (50 mM sodium citrate, pH 5.5) was added to each tube. One μ l of Endo H was added to the Endo H' tube, while the other tube remained a control reaction. Both microfuge tubes were incubated overnight at 37°C, 25 μ l of 2X loading buffer was added, and the tubes were heated at 100°C for 2 min. The total reaction volume was loaded onto a 10% discontinuous SDS-PAGE.

Protein Electrophoresis

Immunoprecipitated proteins were separated by electrophoresis through 9% discontinuous SDS-PAGE gels. Gel apparatuses consisted of vertical slab gel units from either Hoefer Scientific Instruments (model SE 400), or from Bio-Rad® (Protean II model). The gels were prepared several hours before use. Both separating and stacking components of the gel were made from an acrylamide stock solution containing 30% acrylamide and 0.8% *N,N'*-methylenebisacrylamide. The separating component of the gel consisted of a 9% acrylamide solution in 0.375 M Tris, pH 8.8, 0.1% SDS, 0.1% glycerol, and polymerized by adding ammonium persulfate to 0.01% and 0.3 μ l/ml of TEMED. The stacking component of the gel consisted of a 5.4% acrylamide solution in 0.125 M Tris, pH 6.8, 0.1% SDS, 0.05% glycerol, and polymerized by adding ammonium persulfate to 0.006% and 1 μ l/ml of TEMED. Running buffer consisted of Tris-glycine buffer (25 mM Tris, 0.2 M glycine, 0.1% SDS). Prior to loading, samples were vortexed, denatured at 100°C for 3 min, vortexed, and the beads were quickly spun down. Using a 50 μ l Hamilton syringe, 25 μ l of sample was loaded per well. Marker proteins consisted of either Tran-[³⁵S]-labelled VSV protein, or [¹⁴C] methylated protein molecular weight markers (M.W. 14,300-200,000; Amersham Canada Ltd.). Protein gels ran overnight at 2-3 V/cm or several hours at 5 V/cm.

Fluorography was carried out to increase the sensitivity of detection of [³⁵S] labelled proteins. Once protein separation was completed, the gels were removed from the plates, equilibrated with dimethyl sulfoxide (DMSO) for one hour, and impregnated with the scintillant 2,5-diphenyloxazole (PPO; 20% in DMSO) for two hours. Gels were then soaked in water to remove the DMSO. More recently, protein gels were fixed for 30 min in 25% (v/v) isopropanol/10% (v/v) acetic acid, and soaked in Amplify® for 30 min with agitation. Gels were dried in a Bio-Rad® model 483 gel dryer and exposed to Kodak X-OMAT® AR diagnostic film.

ANIMAL IMMUNIZATIONS

The immune response to recombinant adenovirus vectors was studied at two facilities: Animal Facilities at McMaster University Health Sciences Center, Hamilton, Ontario, and at the

Animal Diseases Research Institute, Nepean, Ontario. Preliminary studies were carried out in 6-8 week old female BALB/c mice obtained from Charles River Laboratories, Quebec. All immunizations were carried out in a laminar biological safety cabinet, class II designation, and mice were housed in a Level II isolator unit. Immunization with varying doses of recombinant virus in 100 μ l PBS⁺⁺ was carried out intraperitoneally (IP) using a 26G needle. Mice were checked twice a week, and water, food, and bedding was replaced regularly. Mice were tail vein-bled, or anaesthetized and eye-bled four or eight weeks after the primary immunization. Blood was allowed to clot at room temperature for several hours and at 4°C overnight before the samples were centrifuged and the serum transferred to fresh microfuge tubes.

As Animal Facilities were unable to accomodate large animal work in contained quarters, immunizations of skunks were carried out by the research personnel at the Animal Diseases Research Institute (Agriculture Canada) Nepean, ON. Animal care, immunizations, and blood sampling for antibody determination were carried out by the facility technicians under the direction of Drs. Ken Charlton and Alex Wandeler.

VIRUS NEUTRALIZATION ASSAYS

AdlacZ Neutralization Assays

HeLa cells were plated in 24-well multiwell tissue culture plates (Falcon) at a cell density of 2×10^5 cells per well, and allowed to adhere for several hours. Sufficient wells were set up to include duplicate wells for five dilutions of each serum examined for capacity to neutralize virus, as well as for mock infection (PBS⁺⁺) and several virus dilutions. Serum to be tested was diluted in PBS⁺⁺ by factors of five (1/5, 1/25, 1/125, 1/625, 1/3125): 30 μ l of serum into 120 μ l of PBS⁺⁺, and 30 μ l was then transferred to the next well. Serum dilutions were carried out in 96-well multiwell tissue culture plates. AdlacZ virus stock was diluted to 5×10^6 pfu/ml in PBS⁺⁺, and 120 μ l (6×10^5 pfu) of virus was added to each well of diluted serum. Neutralization reactions were allowed to proceed for one hour at 37°C. Viral dilutions of 3/4, 1/2, 1/4, and 1/8 from the diluted stock (5×10^6 pfu/ml) were also incubated for one hour at 37°C. Medium was removed from HeLa cells, cells were washed once with PBS⁺⁺ and adsorbed

with 200 μ l of test serum plus neutralized virus for one hour at 37°C. Cells were washed once with prewarmed medium (α -MEM containing 10% newborn calf serum, and 1% penicillin/streptomycin) to remove unadsorbed virus. Fresh medium was added and cells were incubated at 37°C. After 24 hours medium was removed and cells were either promptly assayed or were frozen at -70°C until required.

The β -galactosidase assay was carried out in the same multi-well plates. The cell extract was resuspended in 100 μ l of solution A (250 mM Tris, pH 7.8, 1 mM phenylmethylsulfonylfluoride (PMSF stock prepared as 35 mg/ml of ethanol), 0.5% NP40), and the multi-well plate vortexed several times over a period of 20 min at 4°C. Solution B (875 μ l of 10 mM KCl, 1 mM MgSO₄, 100 mM sodium phosphate, 50 mM β -mercaptoethanol, pH adjusted to 7.5) was added to each well and the plates incubated at 37°C for 5 min. Solution C (330 μ l of o-nitrophenyl β -D-galactopyranoside, prepared fresh as 4 g/liter in 100 mM sodium phosphate, pH 7.5) was added, and incubation continued at 37°C for one hour. The reaction was terminated by adding 430 μ l of solution D (1 M Na₂CO₃). Absorbance readings were measured at 420 nm. The blank consisted of the appropriate volumes of all assay reagents. One unit of β -galactosidase activity is equal to o-nitrophenol released per min per 10⁶ cells at 37°C.

VSV Neutralizing Antibody Assays

HeLa cells were trypsinized and plated into 60 mm cell culture dishes to obtain confluent cells the following day. Serum to be tested for neutralizing antibodies was diluted 5-fold (1/5, 1/25, 1/125, 1/625, 1/3125) in PBS⁺⁺ (12 μ l of serum into 50 μ l of PBS⁺⁺). Along with serum from mice inoculated with recombinant viruses, as a control polyserum against VSV Indiana was also included. Dilutions were carried out in 96 well dishes. VSV Indiana HR pp2 virus was thawed on ice, and diluted in PBS⁺⁺ to a final titer of 5 x 10³ pfu/ml calculating 5 x 10² pfu/100 μ l per reaction well. Virus was added to each serum dilution and neutralized at 37°C for one hour. As a control, virus was also diluted in a 10-fold series. Cells were prepared by removing medium and washing once with PBS⁺⁺. 100 μ l of neutralized virus/serum reaction was added per dish of HeLa cells, and non-neutralized virus adsorbed for 1-2 hours at 37°C. Overlay (0.5% agarose, MEM supplemented with 5% newborn calf serum, 1% penicillin/streptomycin as

previously described) was added to each dish. VSV plaques were visible after one day but infections were extended an additional day; cells were fixed with Carnoy's solution for one hour, overlay was removed, and plaques were counted. Neutralizing titres were measured by plaque reduction: a unit was defined as the serum dilution resulting in a 50% reduction in plaque numbers per plate.

Rabies Neutralizing Antibody Assays

Rabies neutralizing antibody determinations were kindly carried out by Dr. Jim Campbell (Department of Microbiology, University of Toronto) according to an ELISA developed by Barton and Campbell (1988) or by a rabies fluorescence inhibition microtest (FIMT) described by Zalan *et al.*, 1979). Purified rabies glycoprotein was used as the antigen in the ELISA assay. Absorbance units from the ELISA assay were converted to FIMT equivalent units (E.U.) from a standard curve (Barton and Campbell, 1988). One FIMT E.U. was defined as the inverse of the highest twofold serum dilution resulting in a reduction of fluorescent foci of ERA strain rabies virus grown under standard conditions by at least-50%. FIMT E.U. were divided by 46 to obtain international units (I.U.).

CHAPTER 3

ADENOVIRAL VECTORS EXPRESSING RABIES GLYCOPROTEIN

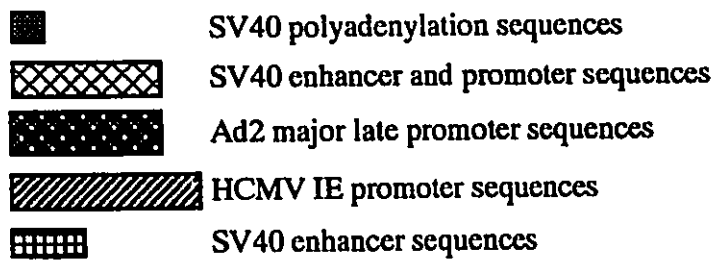
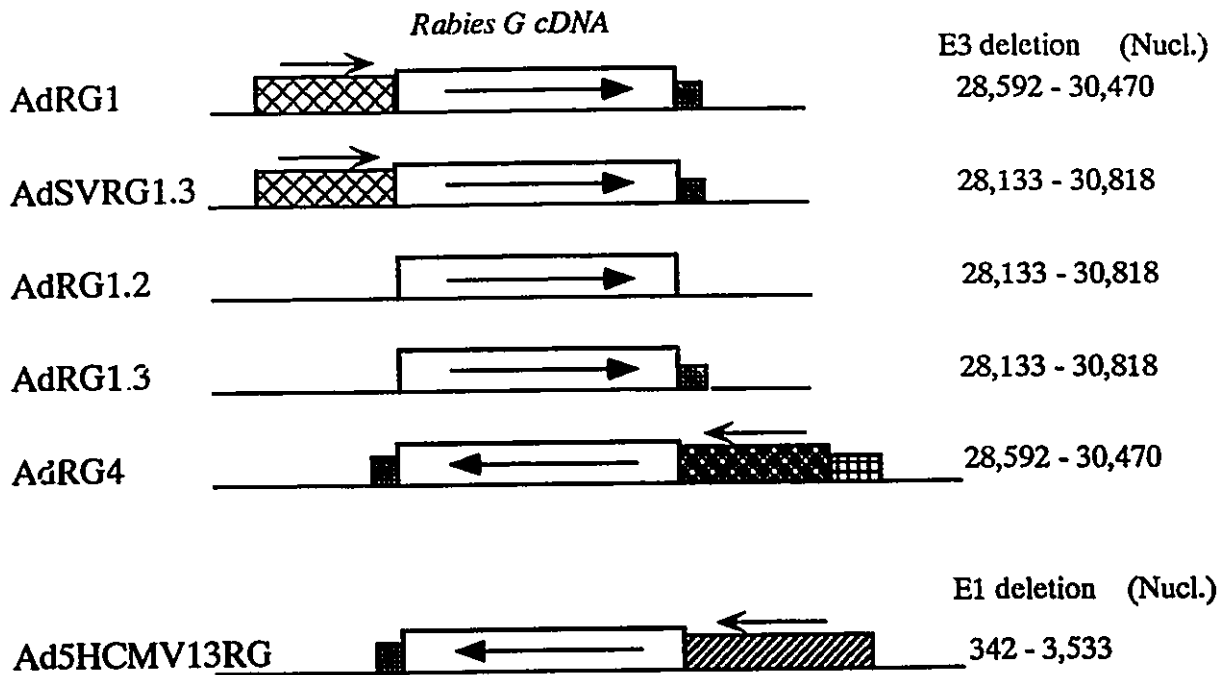
BACKGROUND

The first project was to improve recombinant human adenovirus type 5 (hAd5) vectors expressing rabies glycoprotein (G) for use as wildlife vaccines. Previous studies demonstrated that recombinant hAd5 viruses expressing rabies G or other foreign DNA sequences were effective vaccine vectors (reviewed in CHAPTER 1) although protein expression was not necessarily optimal in those early vectors. It can be argued that increased glycoprotein expression by a vector would allow a lower vaccine dose to induce the same protective immunity. This idea prompted questions concerning the effect of promoter and polyadenylation (polyA) sequences, as well as adenoviral genome deletion size and insert location on foreign protein expression. I examined hAd5 vectors with variations of these parameters for optimized rabies G expression in cell culture and animal models.

Prevec and associates developed AdRG1, a human Ad5 recombinant virus expressing the rabies (ERA strain) glycoprotein gene (Prevec *et al.*, 1990). In this construct, rabies glycoprotein sequences were inserted into the polylinker region of plasmid pSV2X3 between the SV40 early promoter and polyA sequences to generate pSV2RG1. The *Xba*I fragment, containing the SV40 promoter, rabies glycoprotein, and polyA sequences, was transferred into the unique *Xba*I site of pFGdX1 to form pBCRG1. pFGdX1 contains right-end hAd5 sequences with the *Xba*I site marking the deletion of the adenoviral E3 region *Xba*I fragment (79.6 to 84.8 mu). pBCRG1 was cotransfected with *Eco*RI-restricted viral DNA and rescued as recombinant virus AdRG1 (Prevec *et al.*, 1990). AdRG1 expressed very low levels of rabies glycoprotein in cell culture and high virus:cell inputs were required for detection of radiolabelled rabies glycoprotein following immunoprecipitation of cell extracts (R. Spessot and L. Prevec, personal communication).

FIGURE 3.1: RECOMBINANT ADENOVIRUSES CONTAINING RABIES GLYCOPROTEIN SEQUENCES

A pictorial description of constructed hAd5 vectors containing rabies (ERA) glycoprotein sequences. Rabies glycoprotein sequences were placed into the *Bgl*II deletion within E3 (nucleotides (nt) 28,133 to 30,818: AdRG1.3, AdRG1.2, AdSVRG1.3), the *Xba*I deletion within E3 (nt 28,592 to 30,470: AdRG1, AdRG4), or the *Afl*III/*Ssp*I deletion within E1 (nt 342 to 3,533: Ad5HCMV13RG). Rabies glycoprotein expression is directed from the endogenous Ad5 E3 promoter (AdRG1.3, AdRG1.2), or from the exogenous SV40 early promoter (AdRG1, AdSVRG1.3), Ad2 major late promoter (AdRG4), or HCMV immediate early (IE) promoter (Ad5HCMV13RG). SV40 polyadenylation sequences were omitted from AdRG1.2. Arrows indicate the orientation of the sequences (left-right or right-left) in the hAd5 genome.



groups of animals immunized orally, intraperitoneally, or intranasally with AdRG1 elicited good titres of anti-rabies neutralizing antibodies (Prevec *et al.*, 1990; Charlton *et al.*, 1992); however, protective immunization required high doses of the vector.

This chapter describes the construction of recombinant hAd5 vectors containing the rabies glycoprotein gene in combinations with or without exogenous promoters and polyadenylation sequences. Figure 3.1 is a pictorial representation of the recombinant vectors that were constructed. The synthesis of rabies G protein by these vectors was examined *in vitro*. Further, the ability of the vectors to promote an anti-rabies immune response in vaccinated mice and skunks was also investigated.

CONSTRUCTION OF hAd5 RECOMBINANTS CONTAINING THE RABIES GLYCOPROTEIN GENE IN THE E3 REGION

Four hAd5 recombinants containing rabies glycoprotein sequences in the *Xba*I deletion within E3 (AdRG4) or the *Bgl*II deletion within E3 (AdRG1.3, AdSVRG1.3, and AdRG1.2) were constructed (Fig. 3.1).

AdRG1.3

AdRG1.3 was designed to examine rabies glycoprotein expression from the E3 promoter (Fig. 3.1). Beginning with pSV2RG1 which contains rabies glycoprotein sequences between SV40 promoter and polyadenylation sequences (Prevec *et al.*, 1990) I made two modifications, the first was the introduction of a more efficient consensus translation initiation site which allowed a subsequent deletion of the SV40 promoter (Fig. 3.2 A). In pSV2RG1 the rabies glycoprotein sequence lacks a purine nucleoside (guanine or adenine) at a position three nucleotides upstream from the start of the coding sequences. The purine is required to complete a preferred translation sequence (also known as a Kozak sequence: G/ANNATGG; Kozak, 1989). Two *Nco*I sites were identified in the promoter region: one at the SV40 promoter/enhancer boundary (nt -120 to the rabies G start), and the second is at the rabies G translation initiation site (CCATGG). An oligonucleotide linker (AB1185: 5' CATGTCTAGA 3') was designed to

FIGURE 3.2: CONSTRUCTION OF hAd5 RECOMBINANTS CONTAINING RABIES GLYCOPROTEIN

A. pSV2RG1 was cut with *NcoI* and dephosphorylated. Linker AB1185 (5' CATGTCTAGA 3') was phosphorylated, self-annealed, and ligated to linearized plasmid. Bacterial cells were transformed with the ligation mix and plasmid DNA was screened for an additional *XbaI* site replacing a 120 bp *NcoI* fragment. The modifications to pSV2RG1a were confirmed by sequencing with primer AB1180. The 1.8 kb *XbaI* fragment from pSV2RG1a was gel-purified and ligated to *XbaI*-restricted and dephosphorylated pAB14. pOYRG1.3 carries the rabies glycoprotein sequences in the *BglII* deletion within E3 in an orientation parallel to the E3 promoter.

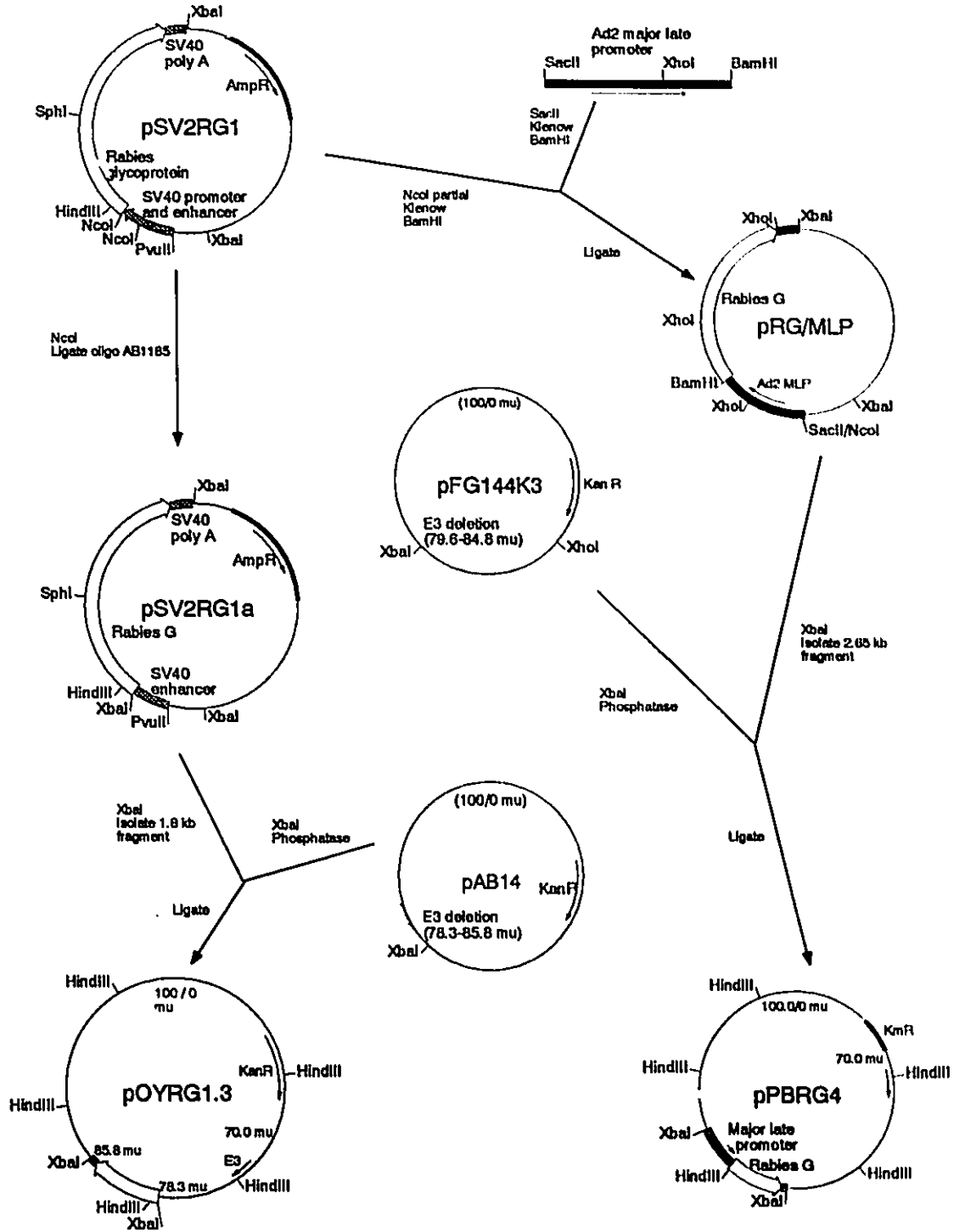
In a second construction, pSV2RG1 was partially cut with *NcoI*, treated with Klenow, and cut with *BamHI*. pKBAAdMLP5a was restricted with *SacII*, blunted with Klenow, then cut with *BamHI*. A 750 bp fragment, containing the Ad2 major late promoter, tripartite leader, and 5' and 3' splice sites, was gel-purified and ligated to linearized pSV2RG1. The 3 kb *XbaI* fragment containing the SV40 enhancer, Ad2 MLP, rabies G, and SV40 polyA DNA sequences, was ligated into *XbaI*/phosphatase-treated pFG144K3 (pPBRG4). pPBRG4 was cotransfected with pFG173 in 293 cells to rescue AdRG4.

B. pSV2RG1 was cut with *XbaI* and a 2.3 kb fragment, containing rabies glycoprotein and SV40 polyA sequences, was gel-purified and ligated into the *XbaI* site of pAB16 in an orientation parallel to the E3 promoter (p16SVRG1.3). p16SVRG1.3 was cotransfected with pFG173 into 293 cells to rescue recombinant virus AdSVRG1.3.

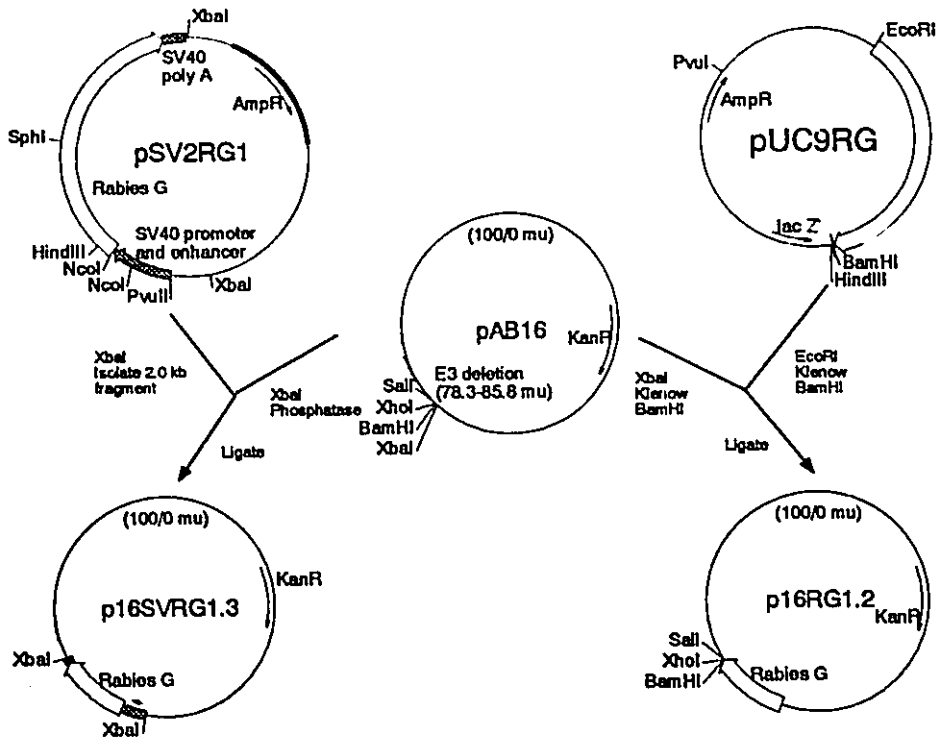
pUC9RG was cut with *EcoRI*, treated with Klenow, and cut with *BamHI*. Linearized plasmid was ligated with *XbaI*-Klenow/*BamHI*-treated pAB16 and kanamycin-resistant plasmid DNA was screened for the presence of 1.7 kb rabies glycoprotein sequences. p16RG1.2 was cotransfected into 293 cells with pFG173 to rescue recombinant virus AdRG1.2.

C. pOYRG1.3 was cut with *XbaI* and a 1.8 kb *XbaI* fragment, containing rabies glycoprotein and SV40 polyA sequences, was gel-purified and ligated into the *XbaI* site of pHCMVsp13 in an orientation parallel to the HCMV IE promoter. The resultant plasmid pDCHCMV13RG was cotransfected with pJM17 and rescued as Ad5HCMV13RG.

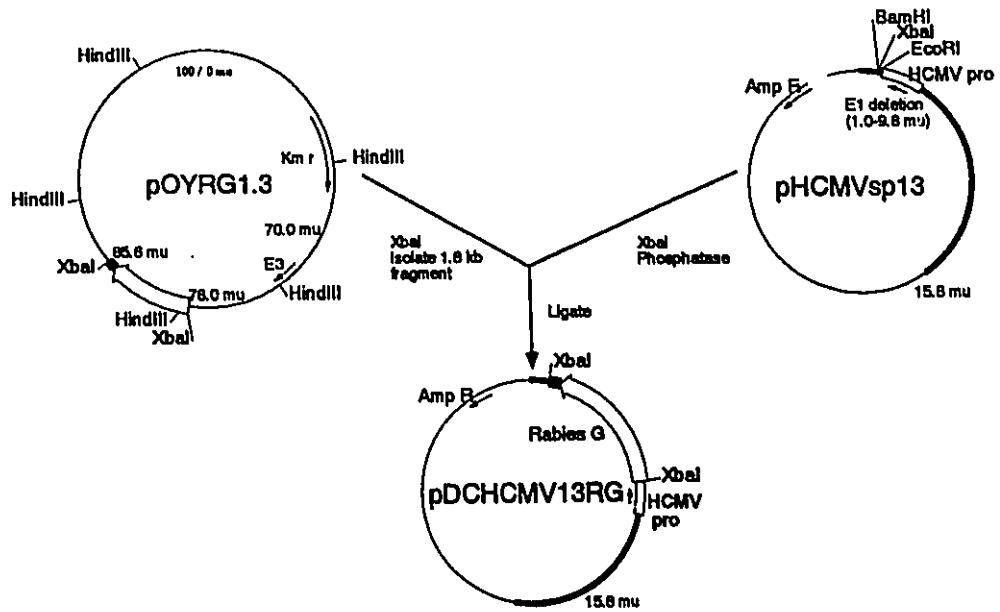
A.



B.



C.



introduce a Kozak consensus sequence and to replace the *NcoI* fragment with an *XbaI* site for simplified subcloning. AB1185 was ligated to *NcoI*-digested pSV2RG1 and the resultant plasmid was called pSV2RG1a. Rabies glycoprotein and SV40 polyA DNA sequences were transferred as a 1.8 kb *XbaI* fragment into pAB14. The resulting plasmid pOYRG1.3 carries the rabies glycoprotein sequences in the *BglIII* deletion within E3 (at 28,133 to 30,818) in parallel orientation to the E3 promoter (Fig. 3.2 A).

AdRG4

The vector AdRG4 was designed to allow rabies glycoprotein expression from an exogenous Ad2 major late promoter. The hAd2 major late promoter, tripartite leader cDNA, and 5' and 3' splice sites were retrieved as a 0.75 kb *SacII/Klenow/BamHI* fragment from pKBAAdMLP5a (Berkner and Sharp, 1984, 1985) and ligated to replace the *NcoI* fragment of pSV2RG1 (Fig. 3.2 A) (P. Brinkley and F.L. Graham, personal communication). As determined by sequencing, an aberrant, but fortuitous, ligation between the Klenow enzyme-treated *BamHI* and *NcoI* sites produced a translation initiation consensus sequence (GACATGG) for rabies glycoprotein in pRG/MLP. The *XbaI* fragment from pRG/MLP, containing SV40 enhancer sequences, Ad2 MLP, rabies G, and SV40 polyA sequences, was inserted into the *XbaI* site of pFG144K3 in an antiparallel orientation to the E3 promoter. pPBRG4 and pFG173 were cotransfected to produce recombinant AdRG4 (Fig. 3.1, 3.2 A). AdRG4 contains SV40 enhancer sequences, Ad2 major late promoter, rabies glycoprotein sequences, and SV40 polyA sequences in the E3 region *XbaI* deletion (nt 28,592-30,470) in an orientation opposite to the E3 promoter (Fig. 3.1).

AdSVRG1.3

The vector AdSVRG1.3 was essentially identical to AdRG1 except that the rabies glycoprotein gene was inserted in the *BglIII* deletion within E3 along with the exogenous SV40 sequences (Fig. 3.1, Fig. 3.2 B). To construct AdSVRG1.3, a 2.6 kb *XbaI* fragment from pSV2RG, containing the SV40 promoter, rabies G, and SV40 polyA sequences, was ligated into the *XbaI* site of pAB16, producing p16SVRG1.3. This plasmid contained a right to left

orientation of the cassette within the E3 deletion (Fig. 3.2 B). Transfection of 293 cells with p16SVRG1.3 and pFG173 produced the vector AdSVRG1.3.

AdRG1.2

To construct vector AdRG1.2, the 1.65 kb *EcoRI*-Klenow/*Bam*HI fragment from pUC9RG, which comprises only rabies glycoprotein coding sequences, was inserted into *Xba*I-Klenow/*Bam*HI-treated pAB16 (Fig. 3.2 B). p16RG1.2 and pFG173 were cotransfected in 293 cells to rescue AdRG1.2. AdRG1.2 does not contain exogenous promoter or SV40-derived polyA sequences, and endogenous adenovirus promoters are required for rabies glycoprotein expression (Fig. 3.1).

CONSTRUCTION OF HAd5 RECOMBINANT CONTAINING THE RABIES GLYCOPROTEIN GENE IN THE E1 REGION

The vector Ad5HCMV13RG contains the rabies glycoprotein gene driven by the human cytomegalovirus (HCMV) immediate early (IE) promoter inserted into an E1 region deletion (Fig. 3.1). pHCMVsp13, used as the starting plasmid for this recombinant, contains left-end sequences of Ad5 (nt 1 to 5,788) with a deletion of the E1 region from nt 342 to 3,533. Within the deletion were inserted the HCMV IE promoter and polylinker sequences (A. Bett and F.L. Graham, personal communication) oriented so that transcription from the HCMV promoter of genes inserted into the polylinker site could be towards the left end of the resultant adenovirus vector. Rabies glycoprotein and SV40 polyA sequences were isolated as a 1.8 kb *Xba*I fragment and ligated into the *Xba*I site of pHCMVsp13 (Fig. 3.2 C). pDCHCMV13RG was cotransfected with pJM17 into 293 cells and rescued as recombinant virus. Since pJM17 is a derivative of the Ad5 mutant *d*/309, the resultant Ad5HCMV13RG carries a small deletion of E3 region sequences which removes both the *Eco*RI (83.6 mu; nt 30,049) and *Xba*I sites (84.8 mu; nt 30,470) (Jones and Shenk, 1978, 1979).

EXPRESSION OF RABIES GLYCOPROTEIN IN CELL CULTURE

I examined the levels of rabies glycoprotein produced by individual vectors by immunoprecipitating rabies glycoprotein from radiolabelled infected cell monolayer. This section presents the results from three independent studies.

Rabies Glycoprotein Expression From The E3 Region

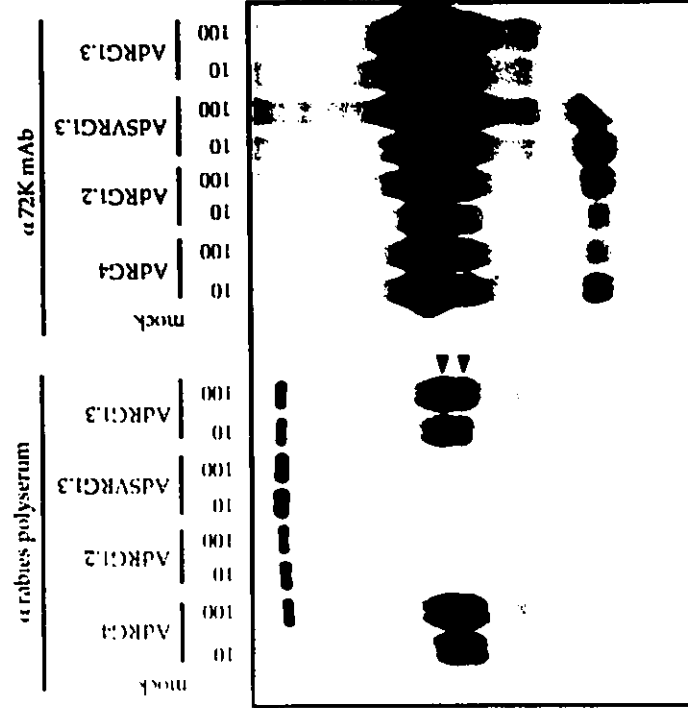
In the first study, I compared rabies glycoprotein expression by AdRG4, AdRG1.2, AdSVRG1.3, and AdRG1.3 to determine whether the differences in these vectors had any affect on the level of glycoprotein expression. HeLa and MDCK cells were infected with either 10 or 100 plaque-forming units (pfu)/cell of each recombinant virus. Rabies glycoprotein was immunoprecipitated from infected cell extracts with rabbit anti-rabies (ERA) polyclonal antiserum (Fig. 3.3 A, B). As seen in Fig. 3.3 A, I detected differentially glycosylated forms of rabies glycoprotein in HeLa cells infected with AdRG4 or with AdRG1.3. The number of species detected reflects the length of the [³⁵S] labelling (two hrs) which allowed detection of glycoproteins throughout the post-translational processing pathway. Both AdRG4 and AdRG1.3 expressed high levels of rabies G while AdRG1.2 produced low amounts of the glycoprotein (Fig. 3.3 A). As seen in Fig. 3.3 B, AdRG4 produced significantly lower levels of rabies glycoprotein in MDCK cells compared to HeLa cells, while AdRG1.2 and AdRG1.3 expressed higher levels of glycoprotein.

Rabbit anti-rabies polyclonal antiserum also immunoprecipitated hexon protein (h), a 103 kD adenoviral structural protein, from infected HeLa cells (Figure 3.3: panel A, top band). Since monoclonal antibodies specific for rabies glycoprotein did not coimmunoprecipitate hexon, the rabbit anti-rabies polyclonal antiserum may display non-specific crossreactivity with the structural protein. Similar results have been reported by others in immunoprecipitations with different rabbit serum (Johnson *et al.*, 1988; Schneider *et al.*, 1989).

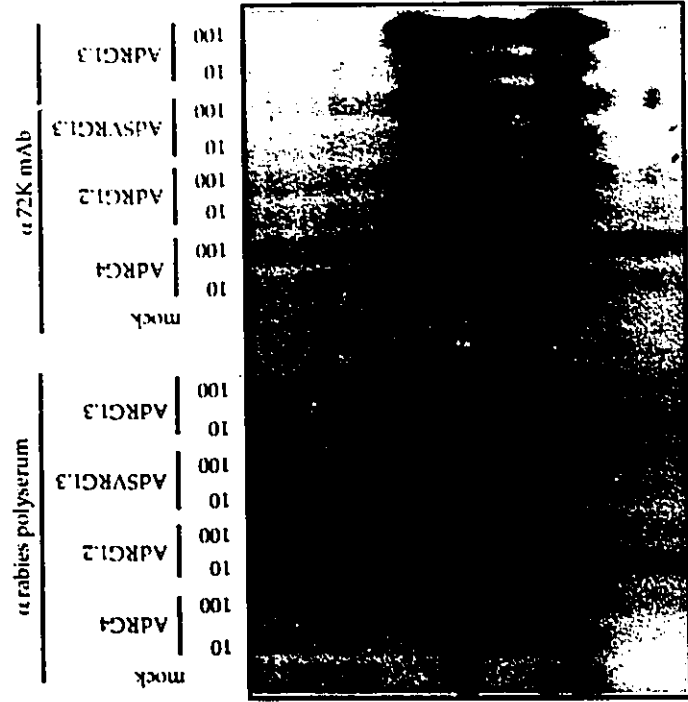
FIGURE 3.3: RABIES GLYCOPROTEIN EXPRESSION IN CELL CULTURE

HeLa and MDCK cells, in 60 mm dishes, were infected at a MOI of 10 or 100 pfu/cell with recombinant vectors AdRG4, AdRG1.2, AdSVRG1.3, or AdRG1.3. Cells were labelled at 17 hr p.i. with 50 μ Ci of Tran[³⁵S] label per dish, and harvested at 19 hr p.i. in RIPA buffer. Rabies glycoprotein was immunoprecipitated with rabbit anti-rabies (ERA) polyclonal antiserum while Ad5 72K protein was immunoprecipitated with MAb H2-19. Arrows identify glycosylated forms of rabies glycoprotein. Hexon protein, a 103 kD adenoviral protein, was immunoprecipitated by a non-specific crossreactivity with the rabbit anti-rabies polyclonal antiserum.

A. HeLa cells



B. MDCK cells



The control immunoprecipitation for adenovirus infection detected the Ad5 72K DNA binding protein (Fig. 3.3 A, B, right side of gel). Densitometry scans of the protein bands in Fig. 3.3 provided a semi-quantitation of 72K and rabies glycoprotein expression. The areas corresponding to the length of the band peak multiplied by maximal band absorbance are presented in Table 3.1. I determined the relative levels of rabies glycoprotein by normalizing to the levels of 72K (rabies G:72K ratio). This calculation allowed a comparison of glycoprotein expression by the four recombinant viruses in cell culture. In HeLa cells, rabies glycoprotein expression by AdRG1.2 and AdSVRG1.3 was approximately 10-fold less than AdRG4 and AdRG1.3. Rabies glycoprotein expression by AdRG4 remained approximately the same in both

TABLE 3.1: RELATIVE EXPRESSION OF RABIES GLYCOPROTEIN

Virus	MOI ⁴	HeLa Cells			MDCK Cells		
		G Area ¹	72K Area ²	G:72K Ratio ³	G Area	72K Area	G:72K Ratio
AdRG4	10	8.92	24.04	0.37	4.92	13.26	0.37
	100	16.00	26.82	0.60	4.67	21.07	0.22
AdRG1.2	10	0.73	18.34	0.04	2.59	5.76	0.45
	100	0.76	19.08	0.04	1.43	18.09	0.08
AdSVRG1.3	10	0.55	23.79	0.02	0.18	9.00	0.02
	100	0.65	25.29	0.03	0.40	17.84	0.02
AdRG1.3	10	5.40	24.8	0.22	10.05	12.91	0.78
	100	10.61	27.43	0.39	22.39	19.89	1.13

¹ Rabies glycoprotein (G) area values expressed were calculated from densitometry scans of autoradiographs as the peak length (mm) x absorbance.

² Ad5 72K protein (72K) area values expressed were calculated from densitometry scans of autoradiographs as the peak length (mm) x absorbance.

³ Relative levels of rabies glycoprotein expression were obtained by the ratio of G:72K.

⁴ Multiplicity of infection, expressed as pfu/cell.

FIGURE 3.4: RABIES GLYCOPROTEIN EXPRESSION BY AdRG1

(facing page)

FIGURE 3.5: RABIES GLYCOPROTEIN EXPRESSION BY AdRG1.3

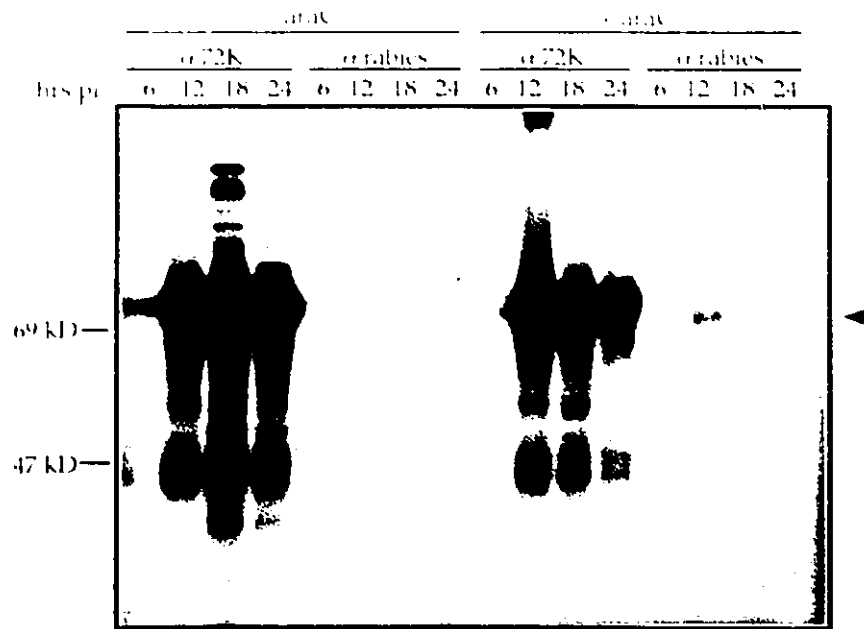
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FIGURE 3.6: RABIES GLYCOPROTEIN EXPRESSION BY AdRG4

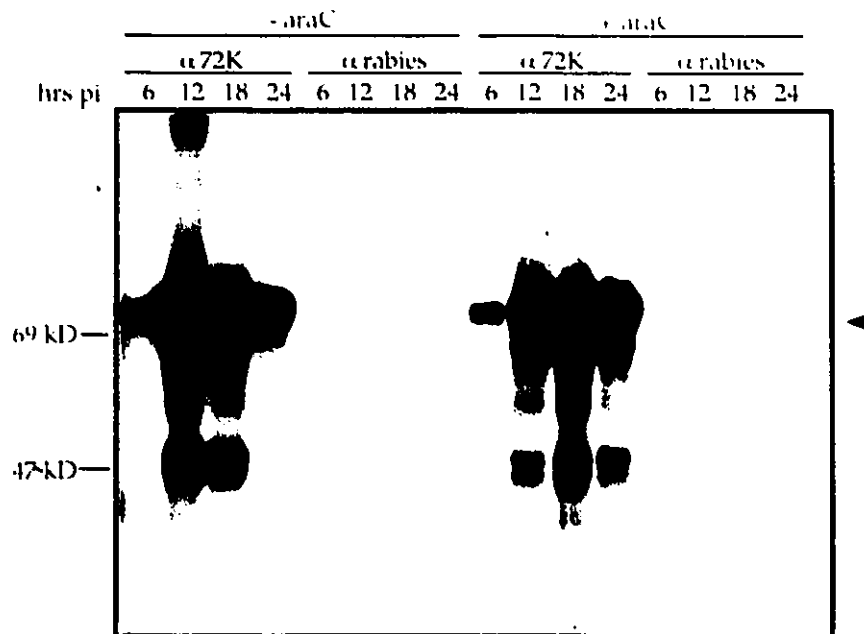
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HeLa and MDCK cells, plated in 60 mm dishes, were infected at a MOI of 20 pfu/cell with recombinant virus. Cells were metabolically labelled with 50 μ Ci of [³⁵S] methionine for 2 hr prior to harvesting at 6, 12, 18, or 24 hrs p.i.. When present (+), araC (cytosine β -D-arabinofuranoside) was added to infected cells at a concentration of 20 μ g/ml of media every 6-7 hrs. Cell extract was immunoprecipitated with MAb 10ED8 2B6 specific for rabies glycoprotein (approx. 70 kD) or MAb specific for adenoviral 72K protein and the 72K degradation product. Note that, in Fig. 3.5, the protein band present in the 6 hr cell extract immunoprecipitated with rabbit anti-rabies polyclonal antiserum is not rabies glycoprotein but rather an overflow of sample from the 24 hr harvest immunoprecipitated with anti-72K serum.

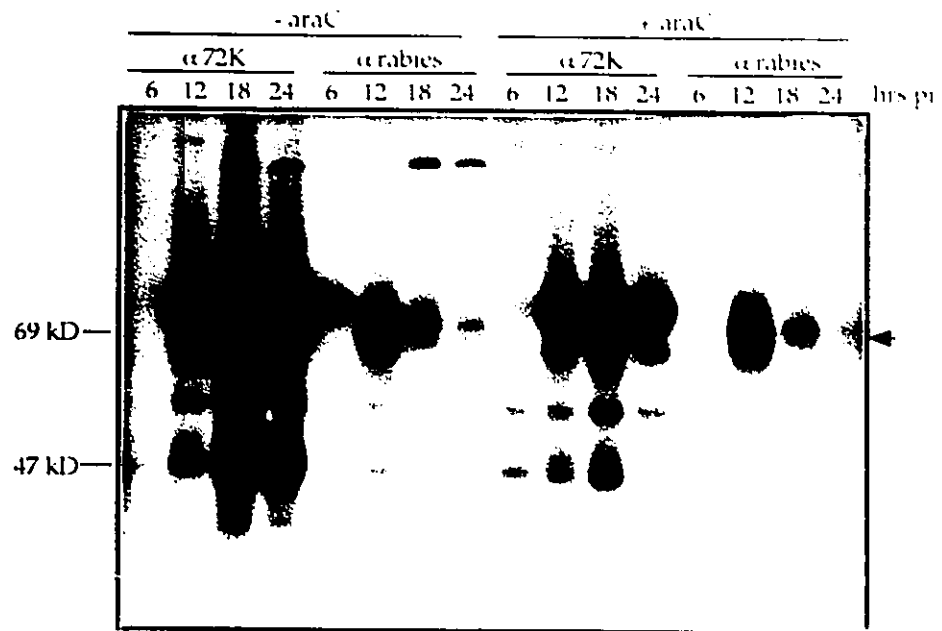
A. AdRG1 infection of HeLa cells



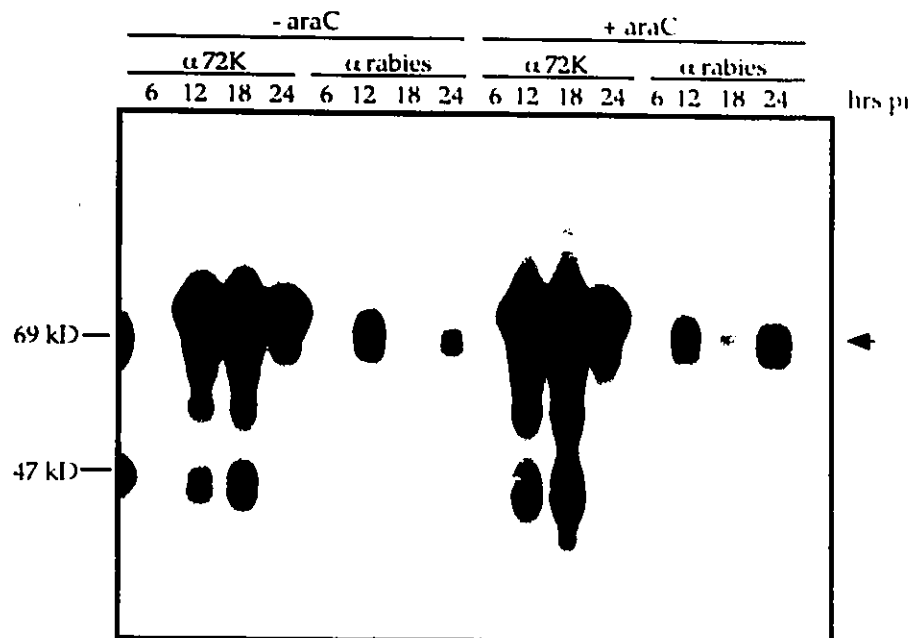
B. AdRG1 infection of MDCK cells



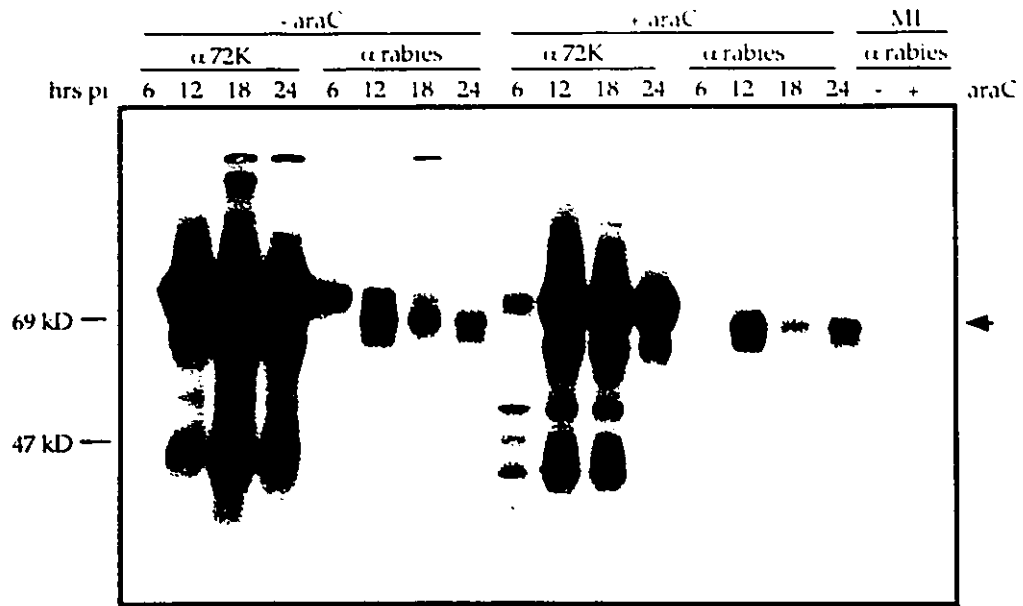
A. AdRG1.3 infection of HeLa cells



B. AdRG1.3 infection of MDCK cells



A. AdRG4 infection of HeLa cells



B. AdRG4 infection of MDCK cells

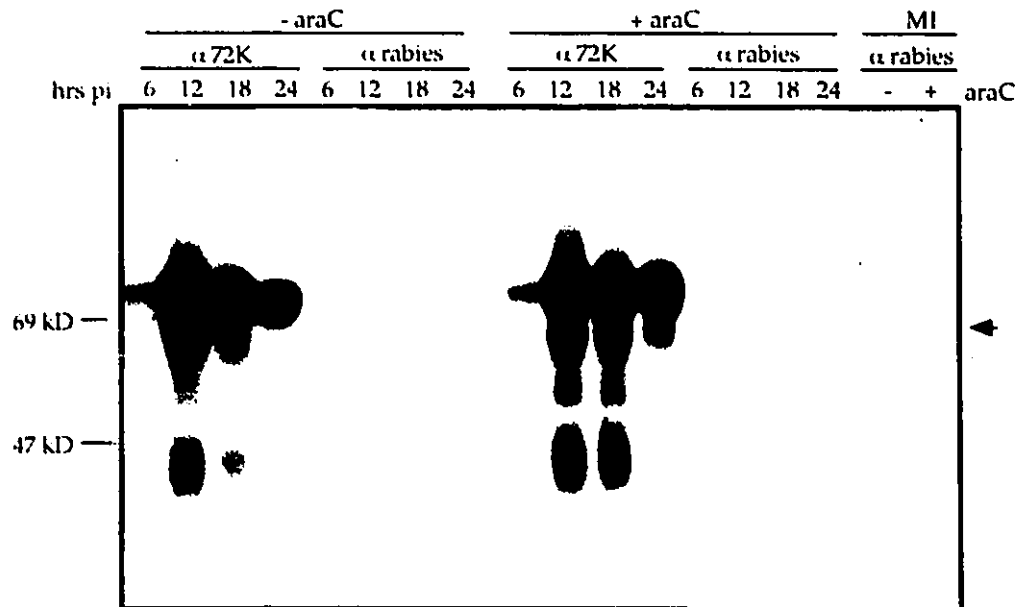
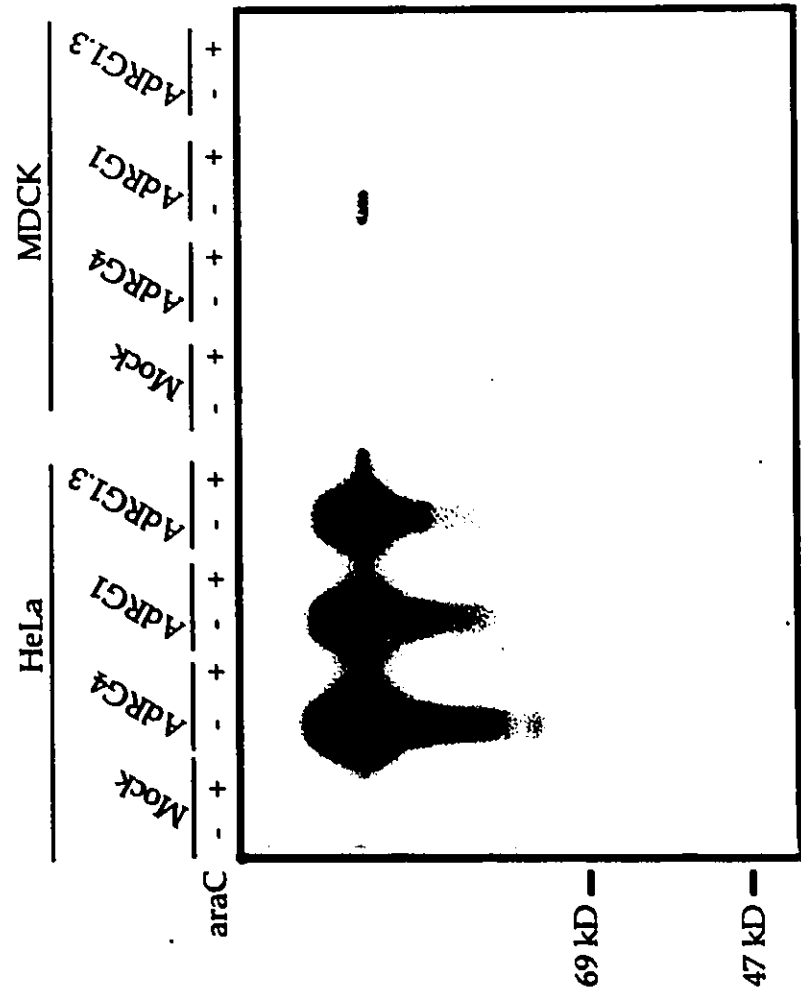


FIGURE 3.7: LATE PROTEIN HEXON IS NOT SYNTHESIZED IN THE PRESENCE OF CYTOSINE ARABINOFURANOSIDE.

HeLa and MDCK cells, plated in 60 mm dishes, were infected at a MOI of 20 pfu/cell with recombinant virus. Cells were metabolically labelled with 50 μ Ci of [35 S] methionine for 2 hr prior to harvesting at 6, 12, 18, or 24 hrs p.i.. When present (+), araC (cytosine β -D-arabinofuranoside) was added to infected cells at a concentration of 20 μ g/ml of media every 6-7 hrs. Cell extracts were immunoprecipitated with MAb specific for hexon protein.



HeLa and MDCK cells, while levels of glycoprotein expressed by AdRG1.3 in MDCK cells were 3-fold greater than in HeLa cells. The level of 72K produced in an infection with AdRG1.2 at a MOI of 10 pfu/cell is low compared to other vectors and is likely due to an inaccurate sample volume. Based on this characterization, I proceeded to examine the temporal expression of rabies glycoprotein by AdRG1, AdRG4, and AdRG1.3.

Temporal Expression Of Rabies Glycoprotein

A time course analysis of rabies glycoprotein expression was carried out in cell lines permissive (HeLa) and nonpermissive (MDCK) for adenovirus replication. In the study I compared recombinant vectors AdRG1, AdRG4, and AdRG1.3. To determine the role of the endogenous major late promoter in rabies glycoprotein expression, the DNA synthesis inhibitor cytosine β -D-arabinofuranoside (araC) was used in some experiments. Since DNA replication must precede late viral mRNA transcription, the addition of araC inhibits expression from the endogenous Ad5 major late promoter. Proteins from infected cell extracts, harvested at 6, 12, 18, or 24 hrs p.i., were immunoprecipitated with MAb specific for either rabies glycoprotein or 72K protein (Fig. 3.4, 3.5, 3.6). Hexon protein, a late protein, was immunoprecipitated with MAb to confirm that late protein synthesis is inhibited in the presence of araC (Fig. 3.7).

Low levels of rabies glycoprotein expression by AdRG1 were detected in HeLa cells at 12 hrs pi while little protein was detected at late times in infection (18 or 24 hrs p.i.) (Fig. 3.4 A). In the presence of araC no significant decrease of rabies glycoprotein was detected (Fig. 3.4 B). Rabies glycoprotein expression early in infection (12 hr p.i.) and in the presence of araC indicated that most of the rabies glycoprotein expression in AdRG1 occurred from the E3 promoter and was not dependent on the Ad5 major late promoter or the exogenous SV40 promoter. Levels of rabies glycoprotein in MDCK cells following infection with AdRG1 were below the level for detection.

As seen in Fig. 3.5, the expression of rabies glycoprotein by AdRG1.3 was significant in both HeLa and MDCK cells. In HeLa cells the level of glycoprotein synthesis decreased from 12 to 24 hours. This result and the fact that araC had no significant effect on the rate of synthesis suggest that the E3 promoter is the primary control element. The kinetics of rabies

glycoprotein synthesis by AdRG1.3 in HeLa cells resembled those observed for AdRG1, although the levels of expression are at least an order of magnitude greater for AdRG1.3. As seen in Fig. 3.5 significant amounts of rabies glycoprotein continue to be synthesized even in the presence of *araC* in MDCK cells at 24 hrs pi (Fig. 3.5 A, B). This result is in agreement with observations made by Grace Martins (M.Sc. thesis, McMaster University, 1991) using an Ad vector carrying a *lacZ* reporter gene and suggests that AdRG1.3 might function well even in animal species which are nonpermissive for adenovirus replication.

As seen in Fig. 3.6 the levels of rabies glycoprotein expression by AdRG4 in HeLa cells was lower than that for AdRG1.3. The reason for this difference with the previous experiment is not known but the experiment served to illustrate the kinetics and relative synthesis in HeLa and MDCK cells by AdRG4. The rate of rabies glycoprotein synthesis from AdRG4 in HeLa cells was maximal at 12 hrs post-infection and decreased by only 50% at 24 hours. This is in marked contrast to AdRG1 and AdRG1.3 which show greatly reduced rates of synthesis at late times. Interestingly, *araC* had relatively little effect on the kinetics of rabies G expression by AdRG4. This result suggests that transcription from the Ad2 major late promoter in this construct is not dependent on DNA synthesis, an observation which will be considered in the DISCUSSION. AdRG4 was expressed much less efficiently at all times post-infection in MDCK cells than in HeLa.

Expression Of Rabies Glycoprotein From Region E1

I examined rabies glycoprotein expression by Ad5HCMV13RG in cell lines that were either able or unable to provide E1 helper function *in trans*. E1 region coding sequences are required *in trans* for Ad5HCMV13RG replication as the virus contains a deletion within E1. Cell lines 293, MRC-5, MDCK, and HeLa were infected with either wild type hAd5 or with recombinant Ad5HCMV13RG at an MOI of 100 pfu/cell. Although an E1 deleted vector of this type can infect all these cell types, it is unable to replicate in all cell lines except for 293. Any detectable rabies glycoprotein expression occurs solely from the HCMV promoter and, in the absence of replication, the levels of glycoprotein expression depend on the MOI. Rabies glycoprotein expression was detected in permissive 293 cells, HeLa, MRC-5, and canine MDCK

cells (Fig. 3.8). As expected the highest levels of expression were obtained with 293 cells (Fig. 3.8) and these levels were approximately 10 to 100 fold higher than levels in cells non-permissive for virus replication. Cells, other than 293, infected with Ad5HCMV13RG, failed to exhibit cytopathic effect for an extended period of time as compared to wild type virus, and would therefore be expected to continue to produce the glycoprotein for extended periods.

RABIES GLYCOPROTEIN EXPRESSION IN INOCULATED MICE

In two separate experiments, female BALB/c mice were immunized intraperitoneally with recombinant vectors AdRG1.2, AdRG1, AdRG1.3, AdSVRG1.3, and AdRG4 to examine rabies glycoprotein antibody in an animal model (Table 3.2). The induction of a protective immune response specific for rabies glycoprotein was measured by the production of rabies virus neutralizing antibodies (VNA). The ELISA protocol detects the total antibody response against rabies glycoprotein, which is a good indicator of the neutralizing response. The results are reported as fluorescence inhibition microtest (FIMT) equivalent unit (E.U.) values for the neutralizing antibody titers. All mice that were administered a high dose of recombinant virus (1×10^8 pfu) produced high titers of rabies neutralizing antibodies (Table 3.2). This included vectors AdSVRG1.3 and AdRG1.2 which had previously yielded very low levels of glycoprotein expression in cell culture. A dilution series of inoculations using AdRG1, AdRG4, and AdRG1.3 was carried out in both studies. A decrease in VNA levels was detected with a decreased dose of vector. At doses of 1×10^7 or 1×10^6 pfu/mouse, I observed moderate antibody levels promoted by the vectors. In the second study, at a dose of 1×10^5 pfu/mouse, only AdRG4 produced an immune response in some animals. Overall, both AdRG4 and AdRG1.3 induced the highest levels of virus neutralizing antibodies. These results support my previous findings that AdRG4 and AdRG1.3 express high levels of rabies glycoprotein *in vitro*. Approximately 32 FIMT (E.U.) correspond to 0.5 IU (international units) of rabies neutralizing antibodies, a level that the World Health Organization officially recognizes as protective against rabies disease development in humans (J. Campbell, personal communication).

FIGURE 3.8: RABIES GLYCOPROTEIN EXPRESSION BY Ad5HCMV13RG

HeLa, MRC-5, 293, and MDCK cells, plated in 60 mm dishes, were infected at a MOI of 100 pfu/cell with wild type Ad5 or recombinant Ad5HCMV13RG. Infected cells were metabolically labelled with [³⁵S] methionine prior to harvesting at either 9 or 25 hrs p.i.. Rabies glycoprotein was immunoprecipitated with MAb 10ED8 2B6.

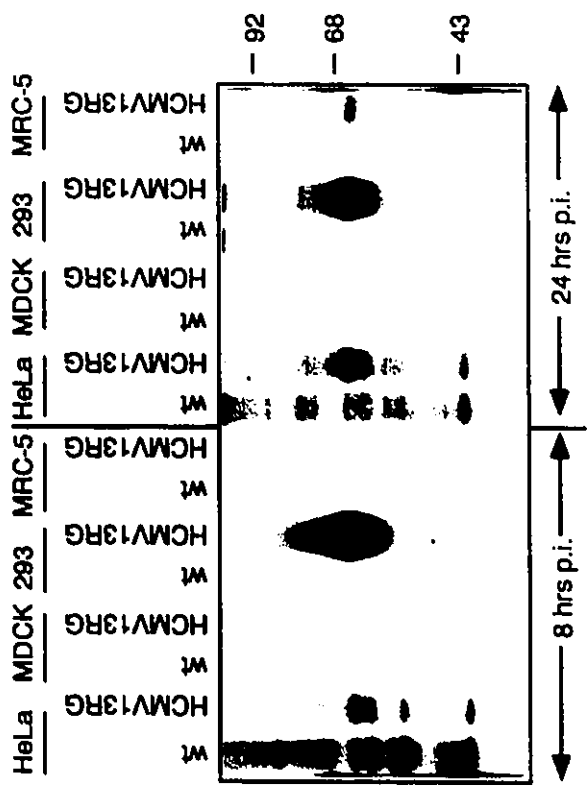


TABLE 3.2: IMMUNIZATION OF MICE WITH RABIES GLYCOPROTEIN VECTORS

Titer ¹	Virus	FIMT (E.U.) Per Individual Animal ²					FIMT (E.U.) Average
		1	2	3	4	5	
Study #1							
1 x 10 ⁸	AdRG1	256	256	256	512	512	358
	AdRG4	512	512	128	512	512	435
	AdRG1.3	512	256	256	256	256	307
	AdSVRG1.3	128	128	512	256	256	256
	AdRG1.2	128	128	128	128	128	128
1 x 10 ⁷	AdRG1	256	256	256	512	512	358
	AdRG4	<8	512	512	512	512	410
	AdRG1.3	16	256	256	256	256	208
1 x 10 ⁶	AdRG1	256	64	128	256	16	144
	AdRG4	64	256	256	16	128	144
	AdRG1.3	128	256	256	128	256	205
1 x 10 ⁵	AdRG1	32	16	16	<8	8	14
	AdRG4	64	64	128	128	<8	77
	AdRG1.3	128	<8	64	<8	128	64
Study #2							
1 x 10 ⁷	AdRG1	32	64	64	8	16	37
	AdRG4	64					64
	AdRG1.3	256	256	128	128	256	205
1 x 10 ⁶	AdRG1	16	<8	8	8	8	8
	AdRG4	16	32	64	128	64	61
	AdRG1.3	64	32	64	16	32	42
1 x 10 ⁵	AdRG1	<8	<8	<8	<8	<8	<8
	AdRG4	64	32	16	32	32	35
	AdRG1.3	<8	<8	<8	<8	<8	<8

¹ Titer of recombinant virus administered IP to BALB/c mice

² FIMT equivalent unit values calculated from ELISA O.D. values.

EVALUATION OF RABIES GLYCOPROTEIN VECTORS IN SKUNKS

Recombinant vectors AdRG1, AdRG4, AdRG1.3, and AdHCMV13RG were evaluated for their ability to induce an immune response in striped skunks (*Mephitis mephitis*). As there are no suitable isolation facilities for large animal studies at McMaster University, the immunizations and serum analysis were carried out by A. Wandeler and fellow researchers at the Animal Diseases Research Institute (Agriculture Canada, Nepean, ON). Skunks were given a 2 ml dose of virus directly into the oral cavity (DIOC). At biweekly or monthly intervals, post-immunization serum was tested for the production of rabies virus neutralizing antibodies (VNA). Within two months of the administration all vectors stimulated the production of VNA to high levels. Tables detailing titers observed in individual animals are presented in APPENDIX 1 and the summary is shown in Table 3.3. As seen in Table 3.3, a response was usually detected by 14 days post-immunization and maximum VNA titers measured at either 30 or 60 days. Several VNA positive animals failed to develop an immune response until day 90. At lower vaccine doses (10^4 - 10^5), nonresponding animals were frequently observed (Table 3.3). There is a partial correlation between the initial dose of vaccine and the calculated VNA titer.

Due to the nonreplicative nature of the AdHCMV13RG vector the first trial of this vector in skunks employed high doses (5.6×10^{10} or 1.8×10^8 pfu of virus). Both doses promoted the development of high VNA titers comparable to titers obtained with doses of 10^5 - 10^7 with the replicative vectors. A study using lower doses of virus is presently in progress (A. Wandeler, personal communication).

TABLE 3.3: IMMUNIZATION OF SKUNKS WITH RABIES GLYCOPROTEIN VECTORS¹

Virus	Amount ¹ Of Virus Given	Responder Total Animals ²	Mean VNA Titer and (Standard Deviation) ³					
			Day 14		Day 30		Day 60	
Study #1								
AdRG1	2.0 x 10 ⁷	7/8	1.1	(2.0)	3.6	(5.0)	10.0	(16.0)
	2.0 X 10 ⁶	7/8	0.3	(0.5)	7.0	(15.4)	12.6	(20.0)
	2.0 x 10 ⁵	2/8	0.0		0.0		0.0	
AdRG4	2.0 x 10 ⁷	7/8	1.6	(2.0)	25.8	(31.3)	43.2	(40.5)
	2.0 x 10 ⁶	8/8	0.2	(0.2)	2.6	(3.9)	2.3	(2.2)
	2.0 x 10 ⁵	2/8	0.0		0.2	(0.5)	0.1	(0.1)
Study #2								
AdRG1.3	3.2 x 10 ⁷	3/4	50.0	(45.9)	58.0	(82.0)	36.2	(40.0)
	3.2 x 10 ⁶	4/4	39.0	(38.0)	44.6	(32.2)	78.2	(75.5)
Study #3								
AdRG1	1.0 x 10 ⁷	7/8	15.8	(30.6)	39.5	(58.8)	36.5	(36.6)
	6.4 x 10 ⁵	8/8	0.9	(2.0)	19.3	(31.8)	19.3	(21.1)
	1.0 x 10 ⁵	3/8	0.0		0.7	(2.0)	2.9	(7.8)
AdRG1.3	2.0 x 10 ⁷	7/8	41.7	(41.5)	133.5	(67.3)	111.3	(62.9)
	3.6 x 10 ⁵	8/8	7.2	(15.1)	19.8	(29.2)	53.5	(60.7)
	6.0 x 10 ⁴	7/8	0.0		1.2	(1.1)	2.0	(1.8)
Study #4								
AdHCMV13RG								
	5.6 x 10 ¹⁰	3/3	12.1	(9.8)	59.3	(25.7)	18.6	(6.5)
	1.8 x 10 ⁸	3/3	1.9	(1.6)	20.4	(21.1)	22.2	(19.3)

¹ Skunks were administered a 2 ml dose of vaccine directly into the oral cavity (DIOC)

² Number of animals developing detectable levels of VNA/ total animals.

³ Mean VNA titer (IU) and standard deviation calculated from all animals immunized with the vaccine. FIMT (E.U.) were divided by 46 to obtain IU.

CHAPTER 4

ADENOVIRAL VECTORS EXPRESSING CHIMAERIC RABIES/VSV GLYCOPROTEIN

BACKGROUND

Rabies and VSV, as rhabdoviruses, have similar genetic and physical compositions (discussed in CHAPTER 1). The envelope glycoprotein of both viruses is required during infection for virus entry into the cells, for the release of virion contents into the cytoplasm, and for the budding of virion particles from the cell. Neutralization of virus with antibodies specific for the glycoprotein effectively prevents viral infection. Rabies and VSV glycoproteins resemble one another in the location of glycosylation sites, the positions of immunogenic domains, and potential disulphide bonds. It can be argued that this similarity leads to comparable glycoprotein conformations. In this project I designed and constructed chimaeric DNA sequences of the two glycoproteins, and tested the model that the hybrid glycoproteins would be translated, correctly processed, and expressed at the cell surface. The immune response generated against the expressed proteins would allow the analysis of immunological epitopes on these proteins.

COMPARISON OF RABIES AND VSV GLYCOPROTEINS

To help in the design of the chimaeric proteins I compared the rabies (ERA strain) and VSV (Indiana serotype) glycoprotein cDNA sequences (Figure 4.1). The cDNA sequences of both cDNAs are similar in length (rabies G cDNA of 1575 nt and VSV G cDNA of 1536 nt) and are translated into precursor proteins of 524 residues (rabies G) and 511 residues (VSV G). With the removal of an amino-terminal signal sequence, the mature rabies and VSV glycoproteins are 505 residues and 495 residues, respectively. Both glycoproteins contain two N-linked oligosaccharide side chains. One N-glycosylation site is located at an equivalent position in the rabies (Asn 338) and VSV (Asn 336) glycoproteins (Fig. 4.1). At the amino acid level, exact

FIGURE 4.1: AMINO ACID SEQUENCE ALIGNMENT OF RABIES (ERA) AND VSV (INDIANA) GLYCOPROTEINS

The amino acid sequences corresponding to the signal sequence, ectodomain, transmembrane, and cytoplasmic domains of both rabies (R) and VSV glycoproteins (G) are compared by the computer program ALIGN followed by a manual screen. The signal sequence is designated with a single line above or below the sequence. The transmembrane region is designated with a double line above or below the sequence. Numbers found above or below sequences correspond to residues in the precursor protein; this format is applied throughout the text. Asterisks indicate homologous residues. Regions showing similarity are as follows: rabies G aa 173-179 and VSV G aa 164-170 (71%), rabies G aa 188-197 and VSV G aa 174-183 (50%), rabies G aa 241-247 and VSV G aa 234-240 (57%), rabies G aa 251-262 and VSV G aa 244-255 (50%), and rabies G aa 316-341 and VSV G aa 314-339 (50%). The amino acid designation is as follows: A, alanine, C, cysteine, D, aspartic acid, E, glutamic acid, F, phenylalanine, G, glycine, H, histidine, I, isoleucine, K, lysine, L, leucine, M, methionine, N, asparagine, P, proline, Q, glutamine, R, arginine, S, serine, T, threonine, V, valine, W, tryptophan, Y, tyrosine.

20 40 60
 R MVPOALLFVPLLVFPLCFGKFPYITILDKLGPWSPIDIHHLSCPNNLVVEDEGCTNLSGF
 * ** * * ** * * * ** * *
 V MKC--LLYLAFL-FIGVNCKFTIVFPHNQGNWKNVPSNYHYCPSSSDLNWHN--DLIGT
 20 40
 80 100 120
 R SYMELKVGYILA IKMNGFTCTGVVTEAENYTNFVGYVTTTFKRKHFRPTDACRAAYNWK
 ** * * * * * * * * * *
 V AIQVKMPKSHKAIQADGWMCHASKWVTTCDFRWYGPKYITQSIRSFTPSVEQCKESIEQT
 60 80 100
 140 160
 R MAGDPRYEESLHNPYPDYRWLRTVKTTKE-SLVII SPSVADLDPYDRSLHSRVFPSGKCS
 * ** ** * * * * * * * * * *
 V KQGT---WLNPGFP-PQSCGYATV-TDAEAVIVQVTPHHVLVDEYTG EWVDSQFINGKCS
 120 140 160
 200 220
 R GVAVSTY CSTNHDIWMPENPRLGMSCDIFTNSRGKRA-SKGSETCGFVDERGL----
 * * * * * * * * * * * * * *
 V -----NYICPTVHNSTTWHS DKVKGL-CDSNLISMDITFFSE GELSSLGKE-GTGFRS
 180 200 220
 240 260 280
 R ----YKSLKGACKLKL CGVLGLRLMDGTWVAMQTSNE-----TKWCPDQLVNLHDFRSD
 * *** * * * * * * * * * * * * *
 V NYFAYETGGKACKMQYCKHWGVR LPSGVWFEMADKDLFAAARFPECPEGSSISAPSQTSV
 240 260 280
 300 320 340
 R EIEHLVVEELVRKREECLDALESIMTTKSVSFRRLSHLRKLVPGFGKAYTI FNKTLMEAD
 * * * * * * * * * * * * * * * * * *
 V DVSLIQDVERILDYSLCQETWSKIRAGLPISPVDLSYLAPKNPGTGPAFTI INGTLKYFE
 300 320 340
 360 380
 R AHYSVRTWNEILPSKGCLRVGGRCHPHVNGVFFNGI--ILGPDGNVLIPE-----MQ
 * * ** * * * * * * * * * * * * *
 V TRYIRVDIAAPILSRMVG MISGTTTERELWDDWAPYEDVEIGPNGVLR TSSGYKFPLYMI
 360 380 400
 400 420 440
 R SLLQOHMELLESSVIPLVHPLADPSTVFKDGDEAEDFVEVHL PDVHNQVSGVDLGLPNW
 * * ** ** * * * * * * * * * *
 V GHGNLSDLHLSSKAQVFEHPHIQDAASQLPDES LFFGDTGL---SKNPIELVEGW FSS
 420 440 460
 460 480 500
 R GKYVLLS-AGALTALMLIIFLMTCCRRVN-----RSEPTQHNL RGTGREVSVT
 * * * * * * * * * * * * * * * * * *
 V WK---SSIASFFFIIGLIIGLFLVLR-VGIYLCIKLKHTKKRQIYTDIEMNRLGK
 480 500
 520
 R PQSGKIISWESHKSGGETRL

alignment of sequence does not extend beyond four adjacent amino acids although stretches of the proteins are similar (Fig. 4.1). According to this alignment (Fig. 4.1), rabies and VSV G may have as many as 10 cysteine residues in common positions. This suggests that comparable disulphide-bonded structures are found in both proteins.

The immunogenic domains on each glycoprotein were summarized in CHAPTER 1. Rabies glycoprotein contains two domains recognized by neutralizing antibodies: domain II maps to residues 53-61 and residues 217-219, while domain III maps to residues 349-359 (Préhaud *et al.*, 1988). VSV glycoprotein contains at least three domains: A2 (residues 50-55), A1 (residues 257-271), and B2 (residues 358-362) (Lefrançois and Lyles, 1982a, 1982b). Rabies G domain III is found in an analogous position to VSV G domain B2, while domain II is in a similar position to the A2 domain. Based on these similarities I predicted similar protein topologies for both rabies and VSV glycoproteins.

OUTLINE OF CHIMAERIC PROTEINS

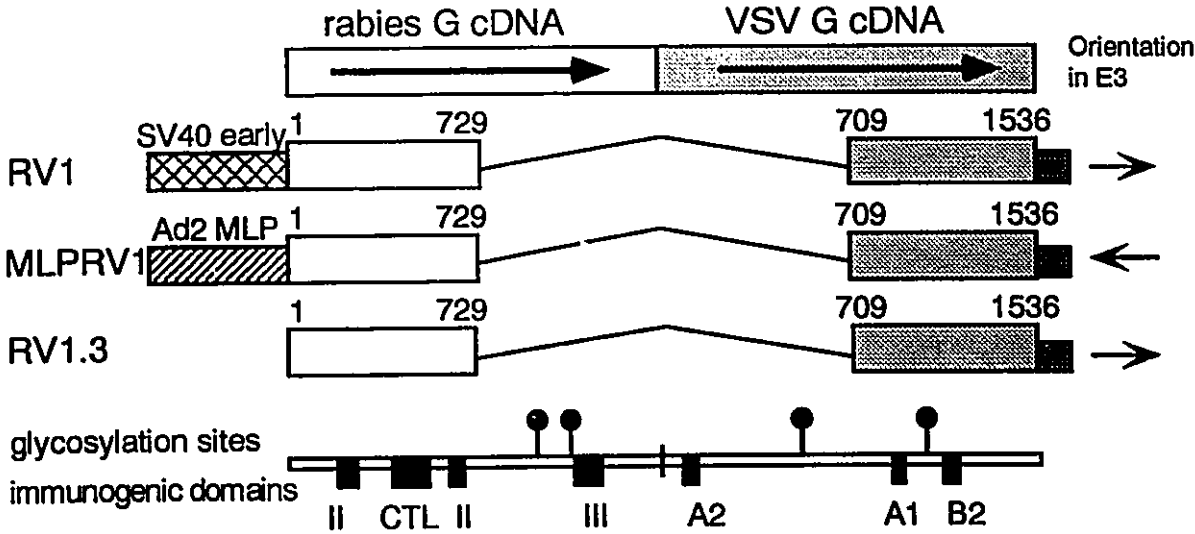
The similarities between rabies and VSV glycoprotein led me to design five chimaeric glycoprotein sequences with assorted combinations of glycosylation sites, cysteine residues, and immunogenic domains (a pictorial representation is found in Fig. 4.2). Protein designation was based on the order of parental protein sequences: RV proteins contain amino-terminus rabies G and carboxy-terminus VSV G sequences whereas VR proteins contain amino-terminus VSV G and carboxy-terminus rabies G sequences. All constructs maintain the translational frame of the parental proteins. The predicted characteristics of the chimaeric proteins are listed in Table 4.1.

In RV1 three residues were aligned from each parental protein (Ala, Cys, Lys: ACK) and corresponded to nt 721 to 729 of rabies G cDNA and nt 700 to 708 of VSV G cDNA. The position of the cysteine residue is conserved among rhabdoviral G proteins and may play a role in protein folding and tertiary structure. The aligned region contains an *SphI* site in the rabies G cDNA sequences which would be utilized in an *in vivo* recombination reaction. RV1 encodes a 504 residue mature protein with one potential N-glycosylation site (Table 4.1), and contains rabies G neutralizing domain II and CTL domain as well as the VSV G A1 and B2 neutralizing domains (Fig. 4.2).

FIGURE 4.2: SUMMARY OF CHIMAERIC RHABDOVIRAL GLYCOPROTEIN CONSTRUCTS

A diagrammatic representation of chimaeric rhabdoviral glycoprotein sequences that were designed based on homologous regions and restriction sites present in the sequence (details in the text). Protein designation was based on the order of parental protein sequences: RV proteins contain amino-terminus rabies G and carboxy-terminus VSV G sequences whereas VR proteins contain amino-terminus VSV G and carboxy-terminus rabies G sequences. All constructs maintain the translational frame of the parental proteins. Numbers above the glycoprotein cDNA boxes indicate nucleotide numbers (calculated from the cDNA sequence) that are present in each construct. The legend found below the constructs indicates potential glycosylation sites (shown as a knob structure) and immunogenic domains (solid boxes). Exogenous promoters (SV40 early promoter, Ad2 major late promoter) and SV40 polyadenylation sequences are represented by smaller, patterned boxes placed on either side of the sequences. Bent lines between cDNA boxes indicate a deletion of sequences.

Rabies - VSV Glycoprotein Recombinants



VSV - Rabies Glycoprotein Recombinants

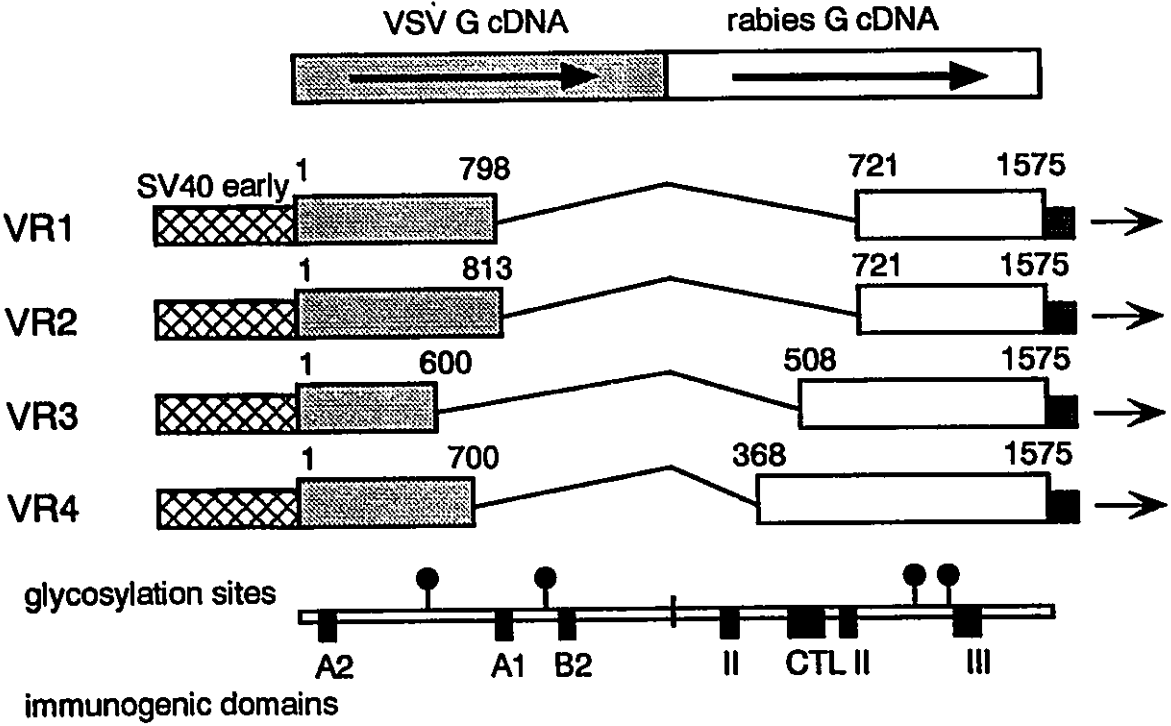


TABLE 4.1: PREDICTED CHARACTERISTICS OF CHIMAERIC GLYCOPROTEINS

Protein	Bases ¹	Precursor ² Protein	Mature ³ Protein	M _w G ₀ ⁴	N-GLY ⁵	M _w G ₂ ⁶
Rabies G	1572 bp	524 aa	505 aa	57,115 D	2	64 kD
VSV G	1533 bp	511 aa	495 aa	55,657 D	2	63.5 kD
RV1	1569 bp	523 aa	504 aa	55,868 D	1	59.5 kD
VR1	1653 bp	551 aa	535 aa	60,098 D	3	70.5 kD
VR2	1671 bp	557 aa	541 aa	60,622 D	3	71 kD
VR3	1671 bp	557 aa	541 aa	60,366 D	3	71 kD
VR4	1908 bp	604 aa	620 aa	69,509 D	3	80.5 kD

¹ Length of cDNA coding sequence (base pairs: bp)

² Length of precursor protein, amino acids (aa)

³ Length of mature protein (aa), after signal sequence cleavage

⁴ Molecular weight (M_w) of the mature protein prior to glycosylation (G₀) calculated from the M_w (daltons; D) of individual residues with a loss of 18 D per peptide bond

⁵ Number of potential glycosylation sites

⁶ Predicted molecular weight of the glycoprotein calculating 3.5 kD per N-linked glycosylation.

VR1 and VR2 contain sequences from the amino-terminus half of VSV G and the carboxy-terminus half of rabies G. In both constructs, the *Pst*I site of VSV G (nt 785) joins the *Sph*I site of rabies G (nt 721) by oligonucleotide linkers. Both chimaeric protein sequences carry three potential N-glycosylation sites (Table 4.1). VR1 and VR2 contain the VSV G neutralizing domain A2 and the rabies G neutralizing domain III (Fig. 4.2). VR2 also contains sequences for the entire A1 domain.

VR3 contains VSV G sequences from nt 1 to the *Nco*I site at nt 600 joined to rabies G sequences from the *Xho*I site at nt 507 to nt 1572. VR3 retains a portion of the VSV G A2 domain, portions of the rabies G domain II and CTL domain, a complete rabies G domain III, and three potential N-glycosylation sites (Table 4.1; Fig. 4.2).

VR4 contains VSV G sequences from nt 1 to the *StuI* site at nt 700 joined to rabies G sequences from the *BstEII* site at nt 368 to nt 1572. VR4 contains a portion of the VSV G A2 domain, a portion of the rabies G domain II, rabies CTL domain and domain III and three potential N-glycosylation sites (Table 4.1; Fig. 4.2).

CONSTRUCTION OF hAd5 RECOMBINANTS CONTAINING CHIMAERIC RABIES/VSV GLYCOPROTEIN

To simplify construction of the chimaeric sequences, pUC-derived plasmids were made which contain rabies and VSV G cDNA sequences in tandem (Fig. 4.3). Rabies ERA glycoprotein cDNA was cloned into the polylinker sequences of pUC8 or pUC9 to generate pUC8RG and pUC9RG. VSV (Indiana) G cDNA was then cloned 5' of the rabies G sequences in pUC9RG (pUC9VR), or 3' of the rabies G sequences in pUC8RG (pUC8RV). Both pUC8RV and pUC9VR were used for the construction of recombinants containing either 5'-rabies-3'-VSV or 5'-VSV-3'-rabies glycoprotein sequences, respectively.

Chimaeric Rabies-VSV Glycoprotein Sequences

Recombinant RV1 was constructed to examine the expression and immunogenicity of a chimaeric protein containing amino-terminal rabies G and carboxy-terminal VSV G sequences. I designed oligonucleotide sequences that complemented *SphI*-treated fragment ends of rabies G cDNA (nts 721 to 726) and were similar to the VSV G cDNA in the region from nt 706 to 723 (Fig. 4.4). The region of homology (16 nt) between linker-based VSV G sequences and plasmid-derived VSV G sequences is of a sufficient length for *in vivo* recombination when the linearized plasmid is transformed into bacterial cells. AB698 and AB699 were ligated to the *SphI*-ends of pUC8RV. *In vivo* recombination between linker-derived VSV G sequences and plasmid-based VSV G cDNA was obtained after the ligation mixture was transformed into *rec*⁺ LE392 cells. Two of 73 bacterial colonies screened contained the correct recombined 4.2 kb plasmid and the recombination event in pRV1 was verified by sequencing (Fig. 4.4). The 1.65 kb *EcoRI* fragment, containing RV1 sequences, was cloned into pSV2X3 (pSV2RV1) and then transferred,

along with SV40 promoter and polyA sequences into pFGdX1 (pOYRV1). pOYRV1 was cotransfected with pFG173 and rescued as recombinant AdRV1.

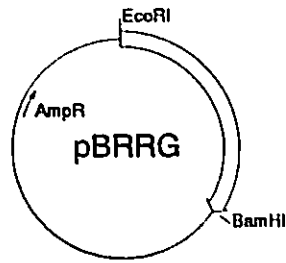
I made modifications to pSV2RV1 to allow me to determine levels of RV1 expression from the E3 or the major late promoters. *NcoI* sites are found both 5' and 3' to the SV40 early promoter. Oligonucleotide AB1185 was phosphorylated, self-annealed, and ligated with *NcoI*-restricted pSV2RV1 to restore a Kozak consensus sequence for rabies G translation and introduce an *XbaI* site 5' of the rabies G sequences for simplified subcloning (pSV2RV1a; Fig. 4.4). The *XbaI* fragment from pSV2RV1a, corresponding to RV1 and SV40 polyA sequences, was cloned into pAB14 (pOYRV1.3; Fig. 4.4). When rescued into virus, AdRV1.3 would allow me to examine RV1 expression from the E3 promoter. I could examine RV1 expression from the Ad2 major late promoter by inserting the *XbaI* fragment into pABMLP to generate pOYMLPRV1 (Fig. 4.4). pABMLP (D. Cummings and L. Prevec, personal communication) contains the Ad2 major late promoter and tripartite sequences cloned as a *SacII/BamHI* fragment into pAB16 at the *XhoI* and *BamHI* sites in opposite orientation to the E3 promoter. pOYMLPRV1 was cotransfected with pFG173 in 293 cells and rescued as AdMLPRV1.

Chimaeric VSV/Rabies Glycoprotein Sequences

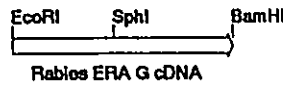
Using pUC9VR as a starting point, I designed recombinant DNA sequences to examine the expression and immunogenicity of chimaeric proteins containing amino-terminal VSV G and carboxy-terminal rabies G sequences. I first attempted to obtain a chimaeric VSV/rabies glycoprotein, complementary to RV1, by *in vivo* recombination between similar sequences of both cDNAs. Residues Ala, Cys, and Lys (rabies G cDNA nt 721 to 726 and VSV G cDNA nt 706 to 723) and the rabies G *SphI* site were used as part of the *in vivo* recombination. Two oligonucleotide linkers (AB592 (5' GAAACTGGAGGCAAGGCATG 3') and AB604 (5' CCTTGCCTCCAGTTTCATA 3')) contained sequences complementary to rabies G *SphI* ends and homologous to the VSV G sequences. The linkers were ligated to *SphI* ends of pUC9VR and the linear plasmid DNA was transformed into LE392 cells to select for *in vivo* recombination. All 387 bacterial colonies screened failed to contain the correct recombinant.

FIGURE 4.3: CONSTRUCTION OF pUC8RV AND pUC9VR

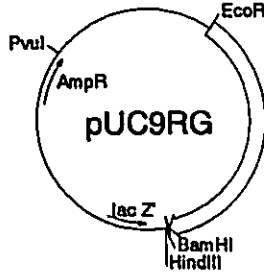
Rabies (ERA) glycoprotein cDNA, a 1.65 kb *EcoRI-BamHI* fragment, was gel-purified from pBRRG and ligated into *EcoRI-BamHI*-restricted pUC9 or pUC8 to generate pUC9RG or pUC8RG, respectively. VSV (Indiana) glycoprotein cDNA, present as a 1.6 kb fragment flanked by both *EcoRI* and *BamHI* sites in pSV2VSVG (P. Lee and L. Prevec, personal communication), was gel-purified as either an *EcoRI* fragment or a *BamHI* fragment. The *EcoRI* fragment was cloned into the *EcoRI* site of pUC9RG 5' and in parallel orientation to the rabies G sequences; this 5.8 kb plasmid was designated pUC9VR. The VSV *BamHI* fragment was ligated into the *BamHI* site 3' and parallel to the rabies G sequences in pUC8RG; the plasmid was designated pUC8RV. Open box represents rabies glycoprotein cDNA while solid box represents VSV glycoprotein cDNA. Orientation of cDNA sequences within the plasmid is designated with the arrows.



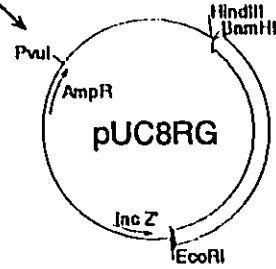
EcoRI, BamHI
Gel purify 1.65 kb
fragment



Ligate with EcoRI-BamHI
restricted pUC9



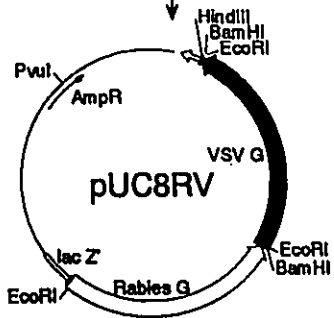
Ligate with EcoRI-BamHI
restricted pUC8



BamHI
Phosphatase

BamHI
Gel purify 1.8 kb
fragment

Ligate



EcoRI
Gel purify 1.8 kb
fragment

EcoRI
Phosphatase

Ligate

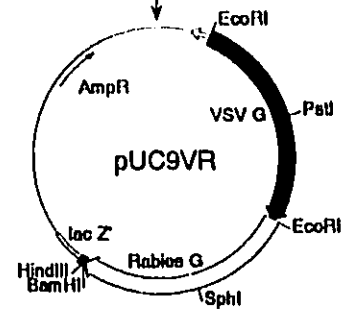


FIGURE 4.4: CONSTRUCTION OF CHIMAERIC RV1 SEQUENCES

pUC8RV was cut with *SphI* and dephosphorylated. Oligonucleotide sequences, with 5' homology to *SphI*-treated fragment ends of rabies G cDNA (nt 721 to 726) and 3' homology to the VSV G cDNA (nt 706 to 723) (AB698 5' CAAAATCAATACTGCAG 3' and AB699 5' GCAGTATTGCATTTTGCATG 3'), were phosphorylated, hybridized in equimolar proportions, and ligated to *SphI*-cut pUC8RV. Ligation mix was transformed into *rec*⁺ LE392 cells to select for *in vivo* recombination between linker-based VSV G sequences and homologous plasmid-based VSV G cDNA. The isolates, both verified by restriction and sequencing analysis, were designated pRV1. pRV1 was restricted with *EcoRI* and a 1.65 kb fragment containing RV1 sequences was gel purified. This fragment was ligated into the polylinker-based *EcoRI* site of pSV2X3 in parallel orientation to the SV40 early promoter element. The resulting 4.5 kb plasmid pSV2RV1 was cleaved with *XbaI* to isolate the larger of two fragments, a 2.3 kb fragment containing the SV40 early promoter, chimaeric RV1, and SV40 polyA sequences. The fragment was ligated into the *XbaI* site of pFGdX1 in parallel orientation to the E3 promoter generating construct pOYRV1 (19.3 kb).

pSV2RV1 was restricted with *NcoI* and dephosphorylated. AB1185 (5' CATGTCTAGA 3'; described in CHAPTER 3) was phosphorylated, self-annealed, and ligated to linearized pSV2RV1. pSV2RV1a, confirmed by sequencing the promoter region, was cut with *XbaI*, and a 1.8 kb fragment was gel-purified and cloned into pAB14 in parallel orientation to the E3 promoter.

The 1.8 kb *XbaI* fragment from pSV2RV1a was gel-purified and ligated into *XbaI*/dephosphorylated pABMLP in an opposite orientation to the E3 promoter generating pOYMLPRV1.

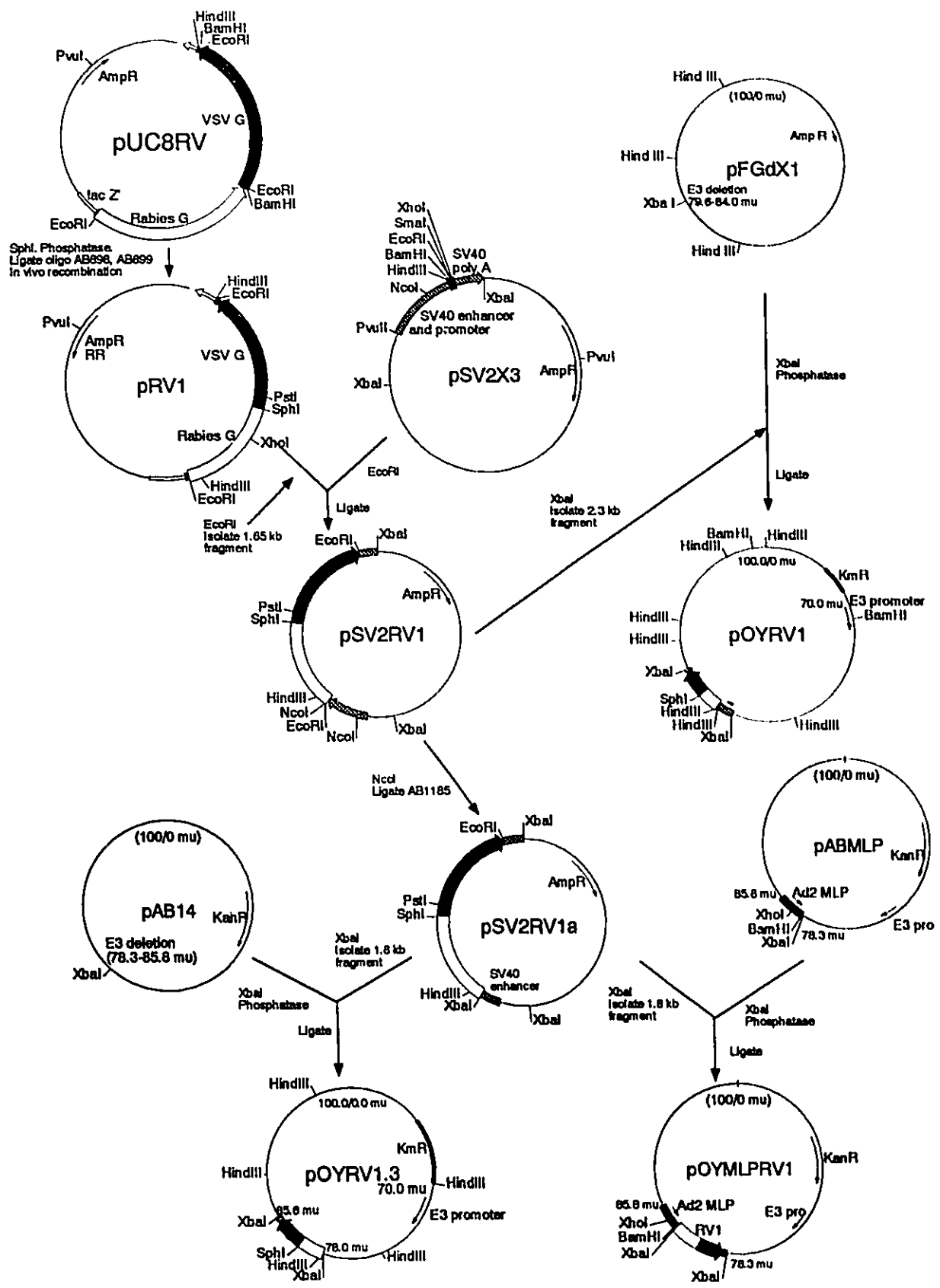
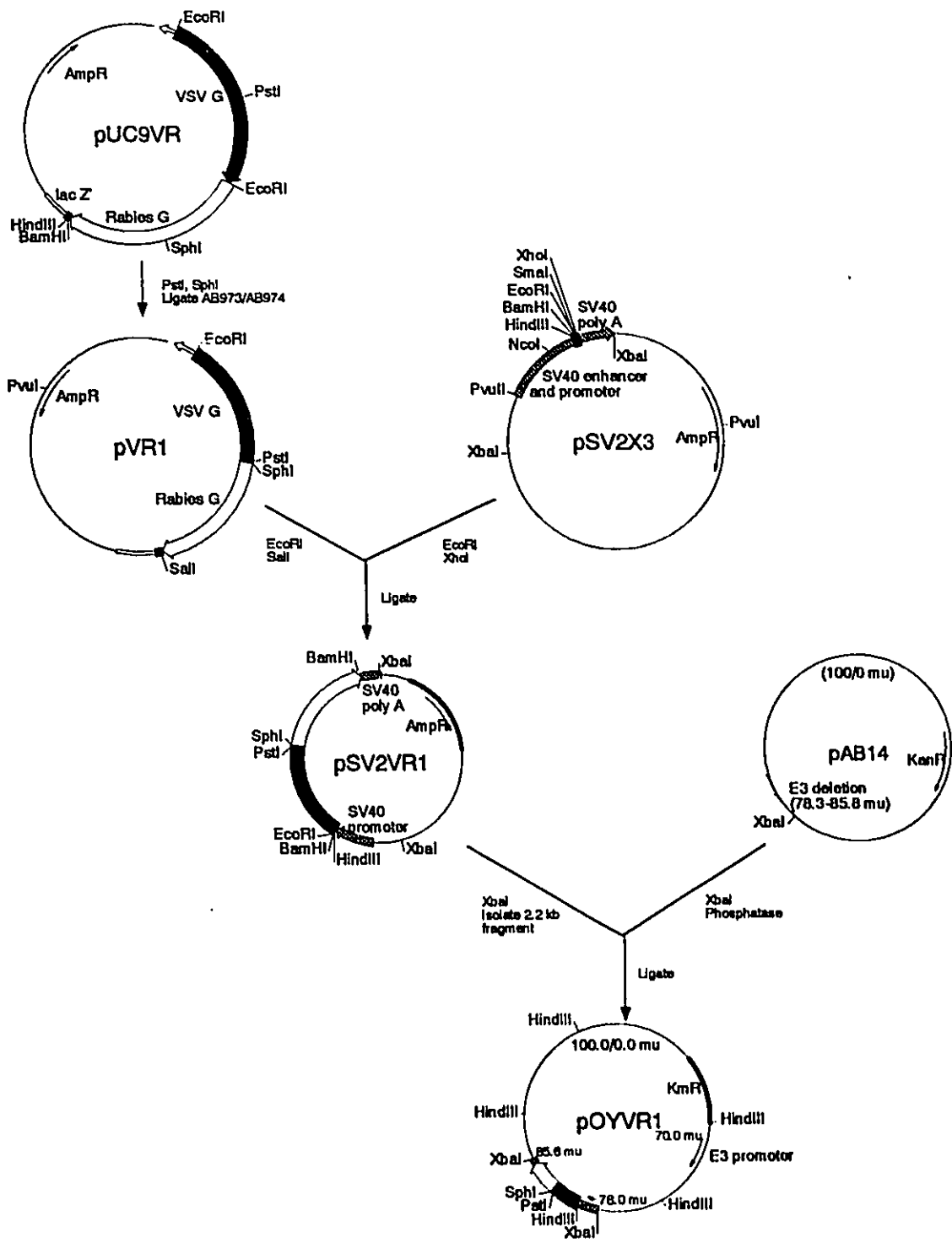


FIGURE 4.5: CONSTRUCTION OF CHIMAERIC VR1 SEQUENCES

pUC9VR was restricted with *Pst*I (nt 785 of the VSV G sequence) and *Sph*I (nt 721 of the rabies G sequence). Oligonucleotides AB973 (5' GCCAGATTCGCATG 3') and AB974 (5' CGAATCT-GGCTGCA 3') were phosphorylated, hybridized in an equimolar ratio, and ligated to linearized pUC9VR. DH5 α cells were transformed with the ligation mix, and colonies were screened for a 4.4 kb plasmid - the resultant plasmid, verified by sequencing, was designated pVR1. The 1.7 kb *Eco*RI/*Sal*I fragment from pVR1, containing VR1 sequences, was ligated with *Eco*RI/*Xho*I-cut pSV2X3 to generate pSV2VR1. pSV2VR1 was restricted with *Xba*I; the 2.2 kb fragment containing VR1 and SV40 polyA sequences was ligated with *Xba*I/dephosphorylated pABI4 DNA (pOYVR1). pOYVR1 was cotransfected with pFG173 in 293 cells and rescued as AdVR1.



In a second approach to generate chimaeric VSV/rabies G sequences I used restriction sites found in analogous positions of the two glycoprotein sequences. The sites were cleaved and modified with enzymes to maintain the correct translational frame. Four chimaeric VSV/rabies glycoproteins were planned with the assistance of linker sequences (Fig. 4.2: VR1, VR2, VR3, VR4). Fig. 4.5 outlines the construction of VR1 although similar manipulations were carried out for the other constructs. In the initial two constructs (VR1 and VR2), pUC9VR was cut at the VSV G *Pst*I site (nt 785) and at the rabies G *Sph*I site (nt 721). Linker sequences contained sequences homologous for both *Pst*I (VSV G cDNA nt 785) and *Sph*I (rabies G cDNA nt 721) ends as well as sequences corresponding to nt 769 to 798 (construct VR1) or nt 769 to 813 (construct VR2) of the VSV G A1 epitope. Linkers AB973 (5' GCCAGATTCGCATG 3') and AB974 (5' CGAATCTGGCTGCA 3') were hybridized and ligated to linearized pUC9VR to construct recombinant VR1, while linkers AB975 (5' GCCAGATTCCTGAATGCCAGAAAGC ATG 3') and AB976 (5'CTTCTGGGCATTCAGGGAATCTGGCTGCA 3') were used to generate VR2. The crossover region in pVR1 and pVR2 was confirmed by restriction and sequencing analysis.

Chimaeric VR3 was designed to delete sequences 3' to VSV G cDNA nt 600 and 5' to nt 508 of the rabies G cDNA. pUC9VR was cut with *Nco*I (nt 595 of VSV G cDNA) and *Xho*I (nt 507 of rabies G cDNA), the ends were filled with Klenow and ligated. After transformation, potential VR3 plasmids were screened for the loss of a 1450 bp fragment and a total pVR3 size of 4.3 kb.

Chimaeric VR4 contains a deletion of sequences 3' to VSV G cDNA nt 700 and 5' to nt 368 of rabies G cDNA. pUC9VR was partially digested with *Stu*I (nt 698 of VSV G cDNA) and digested to completion with *Bst*EII (nt 367 of rabies G cDNA). The resulting *Bst*EII recessed ends were filled in with Klenow enzyme. Plasmids were screened for the loss of a 1220 bp fragment and a total pVR4 size of 4.5 kb.

As was done with pRV1 the chimaeric VSV/rabies sequences were transferred into pSV2X3 in a parallel orientation to the SV40 early promoter and subsequently moved as an *Xba*I fragment, along with SV40 promoter and polyA sequences, into pAB14 and rescued into virus by cotransfection with pFG173.

EXPRESSION OF CHIMAERIC RHABDOVIRAL PROTEINS IN CELL CULTURE

I compared the levels of chimaeric protein expression from the recombinant vectors by immunoprecipitating radiolabelled proteins from infected cell monolayers. The results of three independent studies are presented.

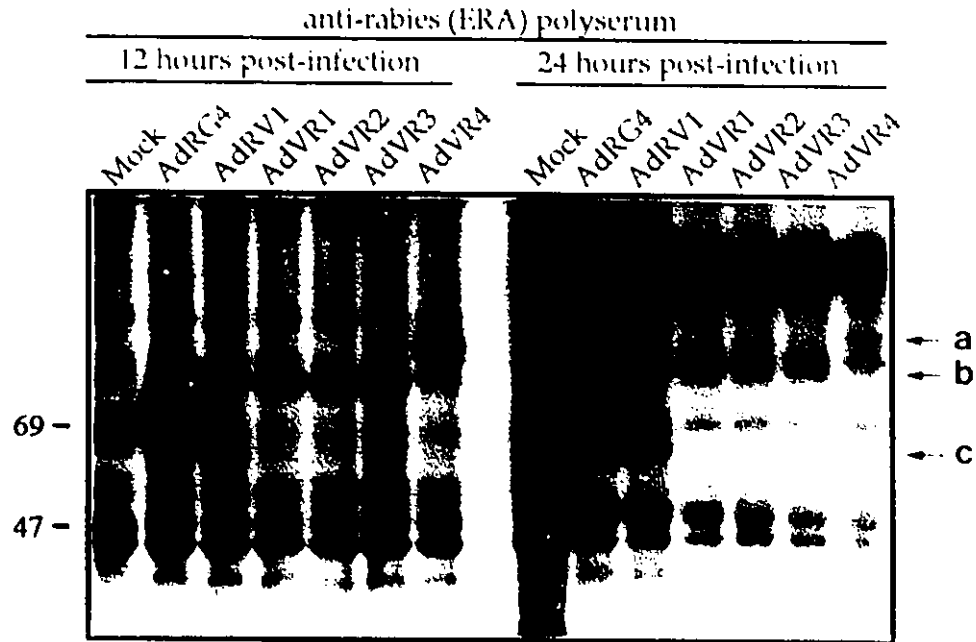
In the first study, I compared chimaeric protein expression by AdRV1, AdVR1, AdVR2, AdVR3, and AdVR4. HeLa and MDCK cells were infected at an MOI of 20 pfu/cell with each vector. As a control for rhabdoviral sequence expression, cells were also infected with AdRG4 and AdG1. Cell extracts were divided approximately in half and immunoprecipitated with rabbit anti-rabies (ERA) polyclonal antiserum (Fig. 4.6 A, C) or VSV (Indiana) (Fig. 4.6 B, D).

Higher levels of chimaeric protein were expressed at 12 hrs p.i. than at 24 hrs p.i. in both HeLa and MDCK cells (Fig. 4.6 A, C). Rabbit anti-VSV polyclonal antiserum precipitated more of the chimaeric products VR1, VR2, VR3, and VR4 than was precipitated with the rabbit anti-rabies polyclonal antiserum (Fig. 4.6, B, D). However, the RV1 product was not detected with rabbit anti-VSV polyclonal antiserum (Fig. 4.6, B, D). Since RV1 is the only recombinant constructed that carries the VSV carboxy sequences I cannot determine whether the failure of the anti-VSV polyserum to precipitate this protein is due to a conformational difference in VSV epitopes in this region or to the absence of antibodies reactive with this portion of the VSV protein. The molecular weights of the chimaeric proteins were estimated by comparing the mobilities of the RV and VR proteins in SDS-PAGE gels to markers of a known molecular weight. A plot of the log (molecular weight of the marker proteins) against protein mobility gave a linear relationship which was used to obtain the molecular weight of the RV and VR proteins. A molecular weight of 61 kD was calculated for protein RV1, which corresponds to the predicted value (Table 4.1) of about 59.5 kD for a glycosylated RV1 molecule. From the gel VR1, VR2, and VR3 were estimated to be approximately 76 kD, which is a slightly higher value than that predicted for properly glycosylated proteins (70.5-71 kD). The VR4 protein had a mobility of approximately 83-84 kD, which was also a higher value than the predicted 80.5 kD for the glycoprotein.

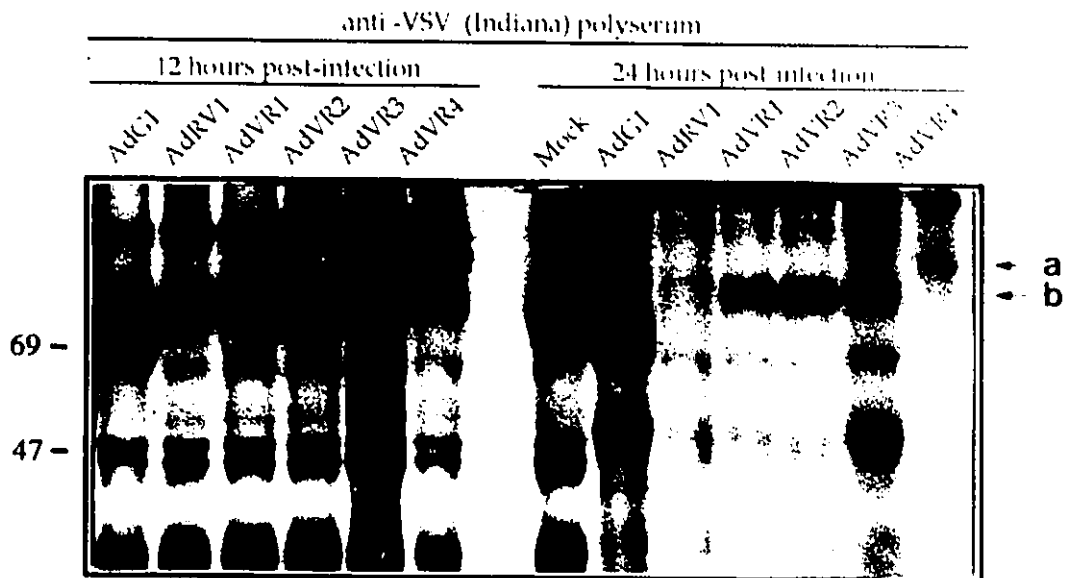
FIGURE 4.6: EXPRESSION OF CHIMAERIC PROTEINS BY RECOMBINANT Ad VECTORS

HeLa and MDCK cells, seeded in 60 mm dishes, were infected with AdG1, AdRG1.3, AdRV1, AdVR1, AdVR2, AdVR3, and AdVR4 at a MOI of 20 pfu/cell. Infected cells were labelled with 45 μ Ci of [35 S] methionine for 90 min prior to harvesting at 12 or 24 hrs p.i.. Chimaeric protein was immunoprecipitated with rabbit anti-rabies or anti-VSV polyclonal antiserum. The molecular weights of the chimaeric proteins were estimated by comparing the mobilities of the RV and VR proteins in SDS-PAGE gels to markers of a known molecular weight. From the gel, RV1 was estimated to be approximately 61 kD, VR1, VR2, and VR3 were estimated as 76 kD, and VR4 protein had a mobility of approximately 83-84 kD.

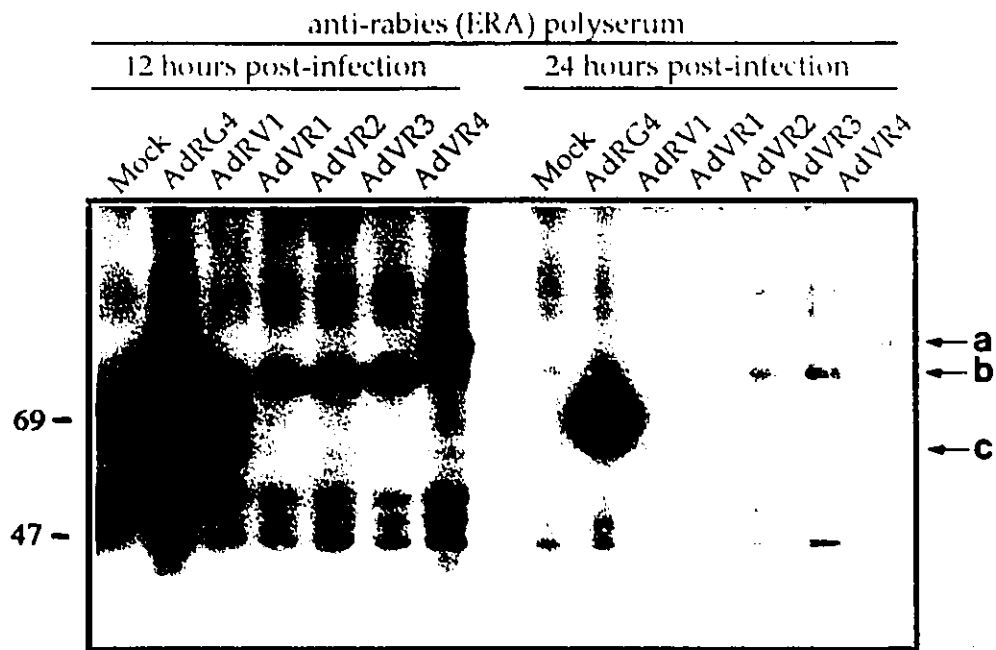
A. HeLa cells



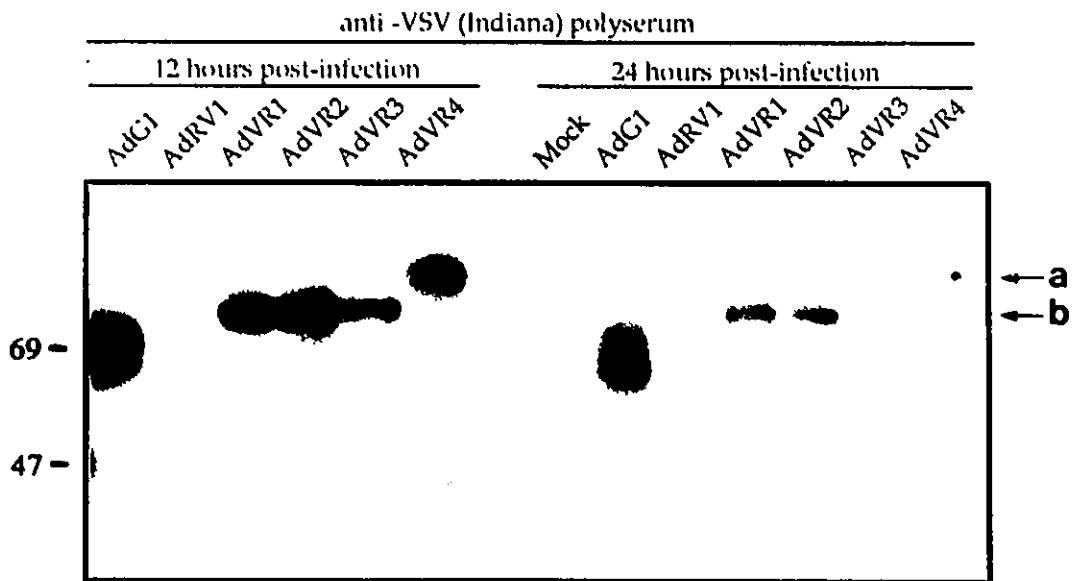
B. HeLa cells



C. MDCK cells



D. MDCK cells



In the second study, I compared RV1 expression from constructs containing the SV40 promoter, the E3 promoter, or the Ad2 major late promoter (Fig. 4.7), and included AdRG1.3 as a control vector. HeLa cells were infected at MOIs of 1, 10, and 50 pfu/cell. Rabies glycoprotein was immunoprecipitated from AdRG1.3-infected cells with MAb, while RV1 was immunoprecipitated with rabbit anti-rabies polyclonal antiserum as the epitope specificity of the anti-rabies G MAb was not known. AdRV1.3 expressed the highest levels of RV1 protein; in this vector RV1 is expressed from the adenoviral E3 promoter (Fig. 4.7). Good levels of expression were also detected for RV1 expression from the Ad2 major late promoter in AdMLPRV1 (Fig. 4.7) although the levels appear to be approximately one-fifth those detected with AdRV1.3. This observation is in agreement with previous results obtained with Ad vectors expressing rabies glycoprotein (see details in CHAPTER 3).

Endo H treatment of immunoprecipitated chimaeric proteins was carried out to determine whether glycosylation groups added to the chimaeric proteins during post-translation were modified through the removal of high mannose groups. The modified proteins would be identified as Endo H resistant, indicating protein transport to the medial Golgi, at least. As the length of radioactive labelling was two hours, it would be expected that some of the control newly synthesized VSV glycoprotein be Endo H sensitive while most of VSV G being Endo H resistant (Fig. 4.8). Both VR1 and VR3 were Endo H sensitive, suggesting a block in protein transport through the post-translational pathway.

IMMUNIZATION OF MICE WITH CHIMAERIC RHABDOVIRAL VECTORS

I examined the immunogenicity of the expressed, chimaeric glycoproteins by administering the recombinant vectors to mice. Vectors were injected intraperitoneally (IP) into female BALB/c mice at an infective dose of 1×10^8 pfu per animal. Typically, 2 or 5 animals were tested per infective dose. After a length of time (usually 4 weeks), blood samples were taken from the animals and serum was tested for the presence of neutralizing antibodies against rabies or VSV. The presence of neutralizing antibodies, *i.e.* antibodies that can neutralize or prevent virus binding and uptake into cells, would indicate that the conformation of the chimaeric

FIGURE 4.7: EXPRESSION OF PROTEIN RV1 BY RECOMBINANT Ad VECTORS

HeLa cells, in 60 mm dishes, were infected with AdRG1.3, AdRV1, AdRV1.3, and AdMLPRV1 at MOIs of 1, 10, and 50 pfu/cell. Cells were labelled with Tran[³⁵S] label (70 μ Ci per dish) for three hrs and harvested at 18 hrs pi. Recombinant RV1 was immunoprecipitated with rabbit anti-rabies polyclonal antiserum while rabies glycoprotein was immunodetected using monoclonal antibody 10ED8 2B8.

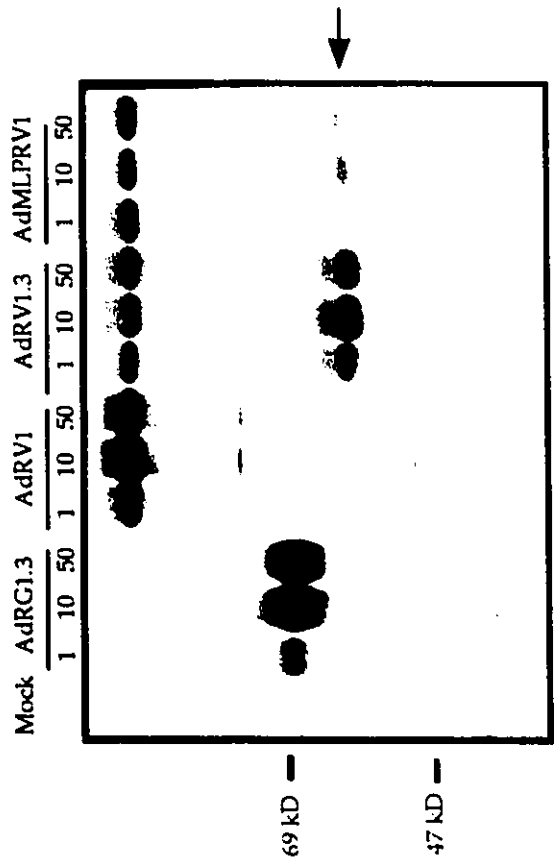
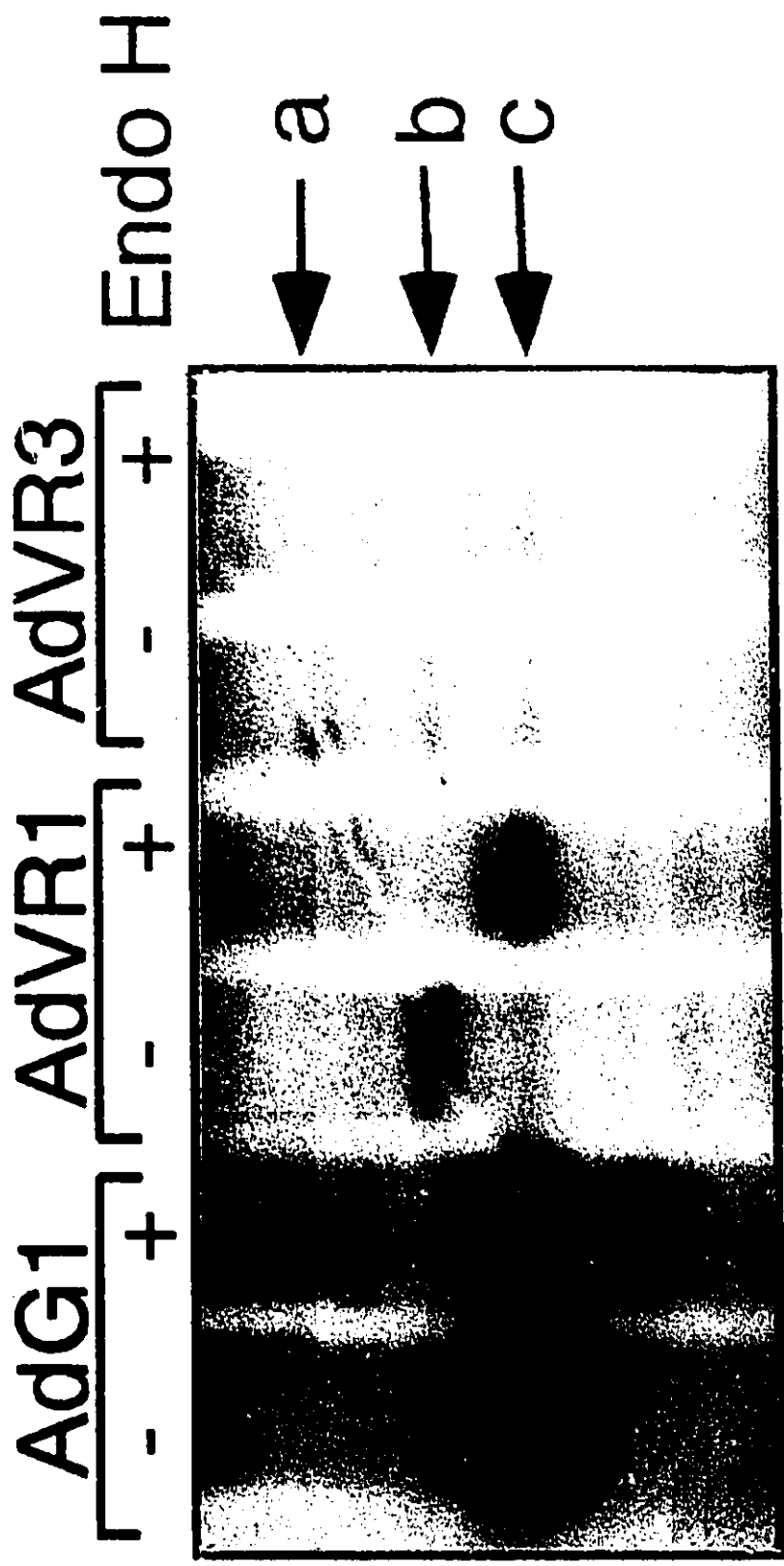


FIGURE 4.8: ENDO H TREATMENT OF CHIMAERIC PROTEINS

HeLa cells, in 60 mm dishes, were infected with AdG1, AdVR1, and AdVR3 at an approximate MOI of 10 pfu/cell. Cells were labelled with Tran[³⁵S] label (150 μ Ci per dish) for 2 hrs at 16 hr p.i.. Proteins were precipitated with a rabbit anti-VSV polyclonal antiserum, and treated with Endo H as described in MATERIALS AND METHODS.



protein resembles that of the parent glycoproteins. Three independent studies are presented (Table 4.2, 4.3, and 4.4).

In the first study (Table 4.2) I gave animals a primary vaccination at week 0, and a secondary, booster vaccination, at 35 days. The booster dose was given in case the primary dose of recombinant vector was insufficient to elicit an immune response and was only able to prime the immune system. Various combinations of AdRV1 as well as the parental viruses AdRG1 and AdVSVG12 were evaluated (Table 4.2). Mice were bled 14 days after the booster dose of virus. A single dose of AdRV1 at week 0 (Table 4.2: animals 9 and 10) or a multiple dose of AdRV1 (Table 4.2: animals 1 and 2) failed to induce neutralizing antibodies against either rabies or VSV. This suggested that the conformation of RV1 does not resemble either rabies or VSV glycoproteins. AdRV1 given at week 0 to animals 3 and 4 was not able to prime the immune system to recognize VSV G expressed by AdVSVG12 (booster dose given in week 5); the serum from these animals had levels of VSV neutralizing antibodies lower than those obtained with AdVSVG12 alone (animal 16). Similarly, AdRV1 given to animals 5 and 6 was not able to prime the immune system to recognize rabies glycoprotein expressed from AdRG1 (booster dose given in week 5). In fact, levels of rabies neutralizing antibodies produced by animals immunized only with AdRG1 (animals 11-13) failed to give a significant anti-rabies response at all. In this case, too much time (a total of seven weeks) may have elapsed from the initial dose with AdRG1 and the specific antibody population was below the levels for detection. In other studies, reported here and by others, higher levels of virus neutralizing antibodies were produced in response both to AdRG1 (Prevec *et al.*, 1990) and to AdVSVG12 (Schneider *et al.*, 1989).

The additional two studies examined the remaining chimaeric vectors (Table 4.3, Table 4.4). In the study presented in Table 4.3, AdVR1 and AdVR3 were compared to rabies glycoprotein expressed by AdRG1.2, while Table 4.4 presents data from a study comparing all recombinant vectors. None of the chimaeric vectors, when administered at an infective dose of 1×10^8 pfu per animal, induced neutralizing antibodies specific for either of the parental glycoproteins. Two of the animals, one given AdRV1 and the other immunized with AdVR1 (Table 4.4), may have shown a small response to the VSV G component of the chimaeric protein.

TABLE 4.2: IMMUNIZATION OF MICE WITH AdRV1

Animal	Virus ¹		Virus Neutralizing Antibodies ²	
	Week 0	Week 5	Rabies	VSV
1	AdRV1	AdRV1	Neg	Neg
2	AdRV1	AdRV1	Neg	Neg
3	AdRV1	AdVSVG12	Neg	1250
4	AdRV1	AdVSVG12	Neg	1250
5	AdRV1	AdRG1	16	Neg
6	AdRV1	AdRG1	16	Neg
7	AdRG1	AdRV1	Neg	Neg
8	AdRG1	AdRV1	Neg	Neg
9	AdRV1	None	Neg	Neg
10	AdRV1	None	Neg	Neg
11	AdRG1	None	Neg	ND ³
12	AdRG1	None	Neg	ND
13	AdRG1	None	8	ND
14	AdRG4	None	4096	ND
15	AdRG4	None	4096	ND
16	AdVSVG12	None	Neg	6250

¹ Mice were immunized IP with an infective dose of 1×10^8 pfu on day 0 and administered a booster of 1×10^8 pfu at 35 days. All mice were tail-bled at 7 weeks post-immunization.

² Serum was analyzed, in two separate assays, for the production of neutralizing antibodies against rabies and VSV. Values for rabies are in FIMT (E.U.), while values for VSV are the reciprocal serum dilutions required to result in a 50% decrease in VSV titer in a plaque assay.

³ ND, not determined.

TABLE 4.3 : IMMUNIZATION OF MICE WITH VSV/RABIES G Ad VECTORS

Animal	Virus ¹	Virus Neutralizing Antibody Titers			
		3 weeks		8 weeks	
		Rabies	VSV	Rabies	VSV
1	AdVR1	Neg	Neg	Neg	Neg
2	AdVR1	Neg	Neg	Neg	Neg
3	AdVR3	Neg	Neg	Neg	Neg
4	AdVR3	Neg	Neg	Neg	Neg
5	AdRG1.2	128	ND ³	64	ND
6	AdRG1.2	128	ND	64	ND
	VSV serum ²	ND	1250	ND	1250

¹ BALB/c mice were immunized IP with an infective dose of 1×10^8 pfu; serum was analyzed 3 and 8 weeks post-immunization.

² Anti-VSV polyclonal antiserum was included as a control serum

³ ND, not determined

TABLE 4.4: IMMUNIZATION OF MICE WITH RABIES/VSV AND VSV/RABIES G Ad VECTORS

Virus ²	Virus Neutralizing Antibody Titers									
	Animal No.									
	1		2		3		4		5	
	R ¹	VSV	R	VSV	R	VSV	R	VSV	R	VSV
AdRV1	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	10
AdMLPRV1	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
AdRV1.3	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
AdVR1	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	50
AdVR2	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
AdVR3	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
AdVR4	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
AdRG1	256	ND ³	256	ND	256	ND	512	ND	512	ND
VSV serum	ND	1250	ND	1250						

¹ Serum was tested for neutralizing antibodies against rabies (R) or VSV.

² BALB/c mice were immunized IP with an infective dose of 1×10^8 pfu.

³ ND, not determined

CHAPTER 5

DISCUSSION

The studies presented in this thesis were carried out with the aim of developing vaccines against rabies virus using human adenovirus type 5 as a vaccine vector. The target populations for the vaccines are wildlife species of North America, such as the raccoon, red fox, and striped skunk. The rabies envelope glycoprotein can induce protective virus neutralizing antibodies (Wiktor *et al.*, 1973; Lafon *et al.*, 1983; Bunschoten *et al.*, 1989) and MHC class I- or class II-restricted cytotoxic T cells (Celis *et al.*, 1985, 1988; Kawano *et al.*, 1990). I used two approaches to obtain high levels of rabies glycoprotein expression in cell culture and improve immune responses in animal species vaccinated with the recombinant adenovirus type 5 vector. In the first approach, described in CHAPTER 3, I varied parameters of the hAd5 vector containing rabies ERA glycoprotein cDNA sequences. In the second approach, described in CHAPTER 4, I attempted to obtain expression and subsequent recognition by the immune system of chimaeric rhabdoviral glycoproteins containing both rabies ERA and VSV Indiana glycoprotein sequences. A discussion and evaluation of the data is presented.

RABIES GLYCOPROTEIN EXPRESSION BY ADENOVIRAL VECTORS

This study examined the effect of introducing exogenous promoters and polyadenylation sequences on rabies glycoprotein expression from adenoviral vectors. The study also attempted to observe the consequences, if any, of deletions made in the adenoviral genome on foreign glycoprotein expression and on the overall characteristics of the viral vector. In general, the expression of a foreign DNA sequence is determined by its genomic and cellular environments, with promoter and enhancer sequences affecting the primary mRNA transcripts, and splice donor and acceptor sequences deciding the mature mRNA species. The expression of foreign inserts from adenovirus vectors will also be affected by endogenous adenoviral promoters and splicing

patterns, both factors that can be influenced by the site of construction and by the deletions created in the adenovirus genome.

To enable comparisons to the original rabies glycoprotein vector, AdRG1 (Prevec *et al.*, 1990), five additional rabies G vectors were constructed: AdRG4, AdRG1.3, AdSVRG1.3, AdRG1.2, and Ad5HCMV13RG. The promoters located directly upstream of the rabies glycoprotein coding sequences included the adenoviral E3 promoter (AdRG1.3, AdRG1.2), the SV40 early promoter (AdRG1, AdSVRG1.3), the Ad2 major late promoter (AdRG4), or the HCMV immediate early promoter (Ad5HCMV13RG). All constructs, with the exception of AdRG1.2, contained SV40 polyadenylation sequences. A Kozak consensus sequence, for improved recognition of the translation initiation codon AUG, was added 5' to the rabies G coding sequences in vectors AdRG1.3, AdRG4, and Ad5HCMV13RG. Cassettes containing the rabies G coding sequences were rescued to replace E1 region sequences from 0.9 to 9.8 map units (mu) (Ad5HCMV13RG), E3 region sequences from 79.6 to 84.8 mu (AdRG1, AdRG4), or E3 region sequences from 78.3 to 85.8 mu (AdRG1.3, AdSVRG1.3, and AdRG1.2).

Rabies glycoprotein expression by all vectors was detected by immunoprecipitation of protein from infections of permissive human cells. The vectors varied both in the amount of rabies glycoprotein expressed and the time frame of expression (early or late with respect to viral DNA replication). A comparison of glycoprotein production by individual vectors showed that levels of expression were affected by the presence of a Kozak consensus sequence at the translation initiation site for the glycoprotein, the stage of infection, and the strength of the adenoviral or exogenous promoters.

The Kozak consensus sequence (G/ANNATGG; Kozak, 1989) places the AUG codon in a favourable context for recognition by the 40S subunit of the ribosome complex. In the absence of a purine molecule (guanine or adenosine) at a position -3 to the AUG codon, a guanine directly after the AUG codon (in a +4 position) is essential for the recognition (Kozak, 1989). Moderate to high levels of expression in cells permissive for adenoviral replication (*i.e.* HeLa or 293 cells) were consistently observed for vectors AdRG1.3, AdRG4, and Ad5HCMV13RG. In each of these vectors, the rabies glycoprotein translation start sequence was modified for efficient translation. The high levels of expression cannot be attributed to the presence of SV40 promoter or enhancer elements as these sequences are absent from AdRG1.3. Rabies glycoprotein

expression by AdRG1, AdRG1.2, and AdSVRG1.3 was not significant unless cells were infected at a high multiplicity of infection. This suggests that the poor level of expression in these three vectors is most likely attributable to the absent Kozak sequences and resulting inefficient recognition of the AUG codon.

I detected high levels of rabies glycoprotein expression at both early (6 hours post-infection) and late (18 hours post-infection) times in permissive infections of human cells using recombinant vectors AdRG1.3 and AdRG4. Both AdRG1.3 and AdRG4 were also able to express rabies G in MDCK cells, a cell line nonpermissive for adenovirus replication and late protein expression. The lack of effect on the levels of glycoprotein expression by AdRG1.3 in the presence of cytosine β -D-arabinofuranoside (araC) suggested that AdRG1.3 rabies glycoprotein transcription occurred primarily from the hAd5 E3 promoter with little or no contribution by the endogenous major late promoter.

The hAd2 major late promoter (MLP) used in AdRG4 corresponds to the cDNA sequences of the MLP promoter region, a tripartite leader ending in the 5' splice site of the third leader, and a 3' splice acceptor site (Berkner and Sharp, 1985). We have also included the SV40 enhancer region upstream of the major late promoter. The enhancer elements typically enable and enhance promoter recognition by cellular transcription factors. The SV40 enhancer element placed in front of the adenovirus type 2 major late promoter was shown to direct a dramatic increase in protein expression (Moreau *et al.*, 1981; Kaufman and Sharp, 1982). The importance of a complete tripartite leader for efficient translation late in infection and maximal expression from the major late promoter was demonstrated by several groups (Thummel *et al.*, 1983; Logan and Shenk, 1984; Berkner and Sharp, 1985; Davis *et al.*, 1985). The presence of viral-encoded VA (viral-associated) RNAs I and II, encoded by DNA sequences adjacent to the major late promoter, prevents the deactivation of the eukaryotic initiation factor 2 (EIF-2). EIF-2 binding increases the levels of translation of tripartite leader-containing late mRNAs (Kaufman, 1985, 1990; Kaufman and Murtha, 1987). The endogenous major late promoter is active within an hour post-infection (Shaw and Ziff, 1980) but, prior to DNA replication, does not contribute to the synthesis of region 3 sequences. The primary late transcript is unable to elongate through early region 3 sequences (Chow *et al.*, 1980) and only the L1 and L2 families of late transcripts,

located upstream of E3, are generated (Shaw and Ziff, 1980; Akusjärvi and Persson, 1981; Nevins and Wilson, 1981).

In this work the exogenous Ad2 major late promoter placed directly upstream of the rabies glycoprotein sequences (construct AdRG4), enabled the production of large amounts of glycoprotein product in cells permissive for adenovirus replication. In cells infected with AdRG4 and treated with araC to inhibit DNA replication, I detected significant amounts of glycoprotein synthesis. Expression in araC-positive conditions may be related to the close proximity of the glycoprotein sequences to the tripartite cDNA leader, *i. e.* in a situation similar to the transcription and expression of the L1 family. Both this effect and the expression of rabies glycoprotein by AdRG4 in nonpermissive MDCK cells may be related to the presence of SV40 enhancer elements upstream of the Ad2 major late promoter. This may be examined by constructing and testing a vector similar to AdRG4 but lacking the SV40 enhancer sequences.

Although the SV40 early promoter is present in constructs AdRG1 and AdSVRG1.3 it appears not to be efficiently utilized as a promoter when placed in parallel to the adenoviral E3 promoter. Mittal *et al.* (1993) showed that expression of luciferase from an Ad5 construct containing the SV40 promoter and luciferase coding sequences parallel to the E3 promoter occurred only after DNA replication suggesting that the SV40 early promoter was not functional and that luciferase expression occurred solely from the major late promoter. Johnson *et al.* (1988) expressed herpes simplex virus (HSV) I glycoprotein B from a vector containing the SV40 promoter in a deletion of E3 region coding sequences. They demonstrated that gB mRNA transcripts in this vector originated from a region upstream of the transcription start site that would be used by the SV40 promoter complexes. In this particular construct Johnson proposed that the SV40 early promoter sequences contained a cryptic splice acceptor site for transcripts originating at the E3 or MLP promoters. A construct containing the HSV 1 thymidine kinase promoter and VSV glycoprotein sequences, in an E3 region parallel orientation, was also found to have transcripts originating upstream of the exogenous promoter (Schneider, 1989; Schneider *et al.*, 1989). Thus, the placement of inserts in parallel to the E3 promoter does not require the presence of exogenous promoters as insert expression will proceed directed from the E3 region or the major late promoters.

This study and others (Wilkinson and Akrigg, 1992; A. Bett and F.L. Graham, personal communication) have shown that the HCMV immediate early (IE) promoter is a suitable exogenous promoter in the expression cassette of foreign sequences inserted into a non-replicating vector. Upstream of the IE promoter are located several 16, 18, 19, and 21 bp imperfect direct repeats which serve as enhancer elements affecting downstream transcription (Boshart *et al.*, 1985). The presence of multiples of enhancer element sequences is known to increase promoter activity (Weber *et al.*, 1984). Along with its strong, constitutive nature that is independent of the adenoviral promoters, the HCMV IE promoter is active in a wide host range (Wilkinson and Akrigg, 1992) which supports its use for protein expression in non-permissive cells.

Using Ad5HCMV13RG we detected higher levels of rabies glycoprotein synthesis in HeLa cells than in MRC-5 cells, although these values were 10 to 100-fold lower than levels detected in permissive 293 cells. Nonpermissive cells infected with Ad5HCMV13RG failed to exhibit cytopathic effect and might be expected, therefore, to retain the rabies glycoprotein-expressing vector for longer periods of time. Using a replication-defective vector with the HCMV promoter replacing E1 sequences Wilkinson and Akrigg (1992) detected high levels of β -galactosidase expression - 27 μ g of protein per 60 mm dish of MRC-5 cells. Expression in different cell types revealed that at 48 hrs post-infection 100% of MRC-5 cells expressed β -galactosidase compared with only 10% of HeLa cells. The study did not quantitatively compare protein expression; further the kinetics of expression at around 48 hrs post-infection may differ from the kinetics observed at the time points in our study.

The vector AdRG1.2 lacked both the SV40 polyA sequences and a translation initiation consensus sequence; consequently, we could not accurately assess the effect of removing the polyA sequence in this construct. It might be expected that constructs with inserted sequences in a parallel orientation to the E3 promoter would make use of either the E3 region polyA sequences or the L5 late family polyA sequences present downstream of the E3 *Xba*I or *Bgl*II deletions. Constructs inserted antiparallel to the E1 or E3 promoters should, as a rule, have polyA sequences included to increase mRNA stability (Zeevi *et al.*, 1982).

CORRELATION OF ANTIGEN EXPRESSION IN CELL CULTURE AND IMMUNOGENICITY IN ANIMALS

This and previous studies (Prevec *et al.*, 1990) found AdRG1 to express rabies glycoprotein at low levels in cell culture. Further, the minimal dose of AdRG1 required for a neutralizing antibody response in mice was measured as 10^4 plaque-forming units (pfu) per animal (Prevec *et al.*, 1990). Hypothetically, lower doses of AdRG1.3 and AdRG4 (*i.e.*, vectors expressing moderate to high levels of rabies glycoprotein in cell culture), would be required for a humoral response in mice. In a dilution series of immunizations (Table 3.2, Study #1) I observed that a dose of 10^5 pfu given to mice produced a 4-5 fold higher mean antibody titer with AdRG1.3 and AdRG4 than with AdRG1. Data obtained in this study has also provided information comparing the effectiveness of the hAd5-rabies glycoprotein vectors in eliciting a humoral response in skunks, a species not protected by the currently licensed vaccine. Both AdRG4 and AdRG1.3 were able to promote neutralizing antibodies in skunks at doses of 2×10^5 or 6×10^4 pfu per animal, respectively (Table 3.3); the minimal dose of these vectors required for a humoral response was 10 to 100-fold lower than the value obtained for AdRG1. These results indicate a correlation between antigen expression in cell culture and immunogenicity in animals.

It has been observed that the doses of AdRG1 packaged into a blister pack bait must be considerably higher than the minimum dose required for direct vaccine administration into the oral cavity of target species. This is to compensate for any viral inactivation occurring in field-placed baits prior to ingestion, as well as for the inefficient delivery of vaccine for the bait into the buccal cavity of the animal. To determine the most effective baiting doses, the levels of rabies glycoprotein expression by AdRG1.3, AdRG4, and Ad5HCMV13RG should be quantitated against AdRG1 in infected cell culture systems that approximate the targetted cells, *i.e.* the mucosal epithelium of the target species.

RABIES GLYCOPROTEIN AND PROTECTION AGAINST DISEASE DEVELOPMENT

The efficacy of recombinant adenovirus vectors in inducing neutralizing antibody responses against rabies glycoprotein and providing protection against disease development was previously documented (Prevec *et al.*, 1990; Charlton *et al.*, 1992). Positive rabies virus neutralizing antibody titers, specific for the rabies glycoprotein and exceeding 0.5 IU/ml, are an indicator of satisfactory seroconversion and are accepted by the World Health Organization (WHO) as a measure of protection against disease development in humans (Nicholson, 1990; J. Campbell, personal communication). Since challenge experiments could not be justified on a cost basis for the skunk model in this study, we accepted that neutralizing antibody levels were good correlates of protection. Previously, cases have been documented whereby animals immunized against rabies disease failed to develop a detectable humoral response and subsequently survived a challenge with live rabies virus. For example, several mice immunized with either 10^4 or 10^5 plaque-forming units of AdRG1 failed to develop neutralizing antibodies at three weeks post-immunization but survived a challenge administered on day 24 (Prevec *et al.*, 1990). Skunks vaccinated with 1.25×10^8 pfu of AdRG1, and foxes vaccinated with 5×10^8 or 4×10^7 pfu of AdRG1 did not develop neutralizing antibodies but survived a rabies challenge at 103 days post-vaccination (Charlton *et al.*, 1992). These results might suggest that a cell-mediated response as well as a humoral response to the glycoprotein are required for disease prevention. Inactivated whole rabies vaccines can induce a strong cell-mediated immune response (Wiktor *et al.*, 1977; Wiktor, 1978), and a challenge of vaccinated animals with street rabies virus results in a secondary cell-mediated response (Wiktor, 1978). A domain associated with cytotoxic T cell activation is localized on the rabies glycoprotein (Macfarlan *et al.*, 1984, 1986; Wunner *et al.*, 1985). In our studies we did not measure cytotoxic T cell activation. Further details of the minimal protective doses of adenovirus vectors could be determined by vaccinating skunks with a dilution series of each recombinant virus and subsequently comparing survival rates after challenge, CTL activity, T helper induction, and antibody titers.

REPLICATIVE-COMPETENT VERSUS DEFECTIVE VECTORS AS VACCINES

This project utilized both replicative-competent and replication-defective Ad5 vectors for protein expression and immunization. High levels of rabies glycoprotein expression are detected from vectors such as AdRG1.3 and AdRG4 in cells permissive for helper-independent adenoviral replication or in non-permissive cells such as MDCK. Similarly, defective vectors such as Ad5HCMV13RG produce high levels of rabies glycoprotein in 293 cells which provide helper function *in trans*. Expression from Ad5HCMV13RG was also observed in cultured HeLa or MRC-5 cells (in which this vector cannot replicate) although the glycoprotein is made at significantly lower levels than in 293 cells.

Observations in non-permissive cell culture systems suggest that it is not necessary to use replication-competent vectors as vaccines for wildlife species. Human adenovirus type 5 is not known to replicate in several of the target species (*i.e.* dogs, skunks) while its effectiveness as a vaccine vector in these species has been repeatedly confirmed (Prevec *et al.*, 1989, 1990; Schneider, 1989; Schneider *et al.*, 1989; Martins, 1991; Charlton *et al.*, 1992). Martins (1991) compared hAd5 replication in human, canine, bovine, and murine cells. Using a replication-competent Ad5 vector with lacZ inserted in the E3 region she showed that β -galactosidase expression was greatly delayed and that Ad5 viral DNA accumulated at very low levels in mouse L cells when compared to an infection of HeLa cells (Martins, 1991). The mouse cell model also showed greatly reduced synthesis of several late structural proteins (Eggerding and Pierce, 1986; Martins, 1991). In canine cells there was little effect on β -galactosidase expression though total viral DNA synthesis was reduced 5-fold and almost no detectable late structural proteins were observed (Martins, 1991). These results suggest that the ability of the vector to replicate in the target species is not an important factor of the vaccine provided that expression of the antigen is independent of viral DNA replication and late protein synthesis. This independence can be attained by using exogenous promoters which are active in the target species or by relying on expression from Ad5 early promoters. In addition, the non-replicative nature of the defective vectors increases the safety and acceptability of the vectors as wildlife vaccines by reducing the risks of horizontal and vertical transmission between individual animals and risks associated with accidental contact with the human population.

USE OF REPLICATION-DEFECTIVE VECTORS IN GENE THERAPY

Nonreplicative adenovirus vectors expressing foreign genes are being applied as tools in gene therapy to overcome known single-gene deficiencies in somatic cell populations (Jaffé *et al.*, 1992; Rosenfeld *et al.*, 1992; Stratford-Perricaudet *et al.*, 1992; Le Gal La Salle *et al.*, 1993; Mastrangeli *et al.*, 1993; and others). The vectors can only be propagated if adenovirus E1 proteins are provided *in trans* (*i.e.* propagation on 293 cells or in the presence of helper virus); once introduced into deficient cell populations the vector will express the foreign protein while failing to produce progeny virus. The adenovirus genome, maintained as an episomic DNA molecule, does not contribute to cytopathic damage (Levrero *et al.*, 1991; Wilkinson and Akrigg, 1992; Le Gal La Salle *et al.*, 1993; A. Bett and F.L. Graham, personal communication; this work) while the absence of a lytic infection contributes to higher levels of protein expression over a longer period (Levrero *et al.*, 1991). Using a replication-defective vector expressing β -galactosidase to infect rat nerve cells, Le Gal La Salle *et al.*, (1993) detected protein activity *in vivo* in microglial cells, astrocytes, and neurons as short as 24 hrs pi; expression continued for two months though with a decrease in overall activity. A vector expressing the normal human cystic fibrosis transmembrane conductance regulator (CFTR) is undergoing preliminary tests for therapy in cystic fibrosis (CF) (Rosenfeld *et al.*, 1992). *In vitro* this vector was able to correct the CF defect in pancreatic cells homozygous for the common $\Delta F508$ CF mutation, while *in vivo* studies in lung tissue of cotton rats demonstrate *in situ* CFTR expression and the detection of normal CFTR mRNA up to 6 weeks post-infection (Rosenfeld *et al.*, 1992). A potential drawback of nonreplicative adenovirus use in gene therapy of mucosal tissues may be encountered if a concurrent wild type adenovirus infection is contracted - this could result in the replication of the therapeutic vector by complementation.

FUTURE PROSPECTS OF HUMAN ADENOVIRUS VECTORS EXPRESSING RABIES GLYCOPROTEIN AS A WILDLIFE VACCINE SYSTEM

This study has confirmed previous positive findings concerning rabies glycoprotein expression by human adenovirus vectors in a vaccination scenario. The Ad5-rabies glycoprotein

vectors can be used to immunize mice and skunks by several routes, including by direct installation into the oral cavity. The efficacy and safety record of AdRG1 has been proven both in target species and non-target species (such as squirrels and groundhog) (A. Wandeler, personal communication) and will likely be as good or better for AdRG1.3, AdRG4, and AdHCMV13RG. A clear advantage of Ad5-rabies glycoprotein vaccines as compared to vaccinia-rabies glycoprotein is the efficacy in skunks, a problematic population of disease carriers especially in urban centers (A. Wandeler, K. Charlton, and others, Agriculture Canada, personal communication). The prospects for these vectors as wildlife vaccines for Canada are, indeed, quite promising and should be further pursued.

EXPRESSION OF CHIMAERIC RHABDOVIRAL GLYCOPROTEINS

I and others have used adenovirus vectors to express viral membrane proteins in cultured cells and in animal models. We have demonstrated that expression of the foreign proteinS within the targeted animal species stimulates a protective antibody response against infection with the parent virus (such as rabies). The feasibility of constructing a subunit vaccine against rabies, using defined glycoprotein peptides, has been discussed (Wunner *et al.*, 1983). It is difficult to define suitable B or T cell antigenic determinants to be included in the subunit vaccine without knowledge of the crystal structure of the entire protein molecule (Laver *et al.*, 1990). Attempts to identify B cell epitopes have relied on techniques to predict regions of hydrophilicity, regions of solvent accessibility, atomic mobility, and secondary α helix and β turn structure (Scheidtmann, 1989; Laver *et al.*, 1990). T cell epitopes have also been predicted using various models including an amphipathic α helix (DeLisi and Berzofsky, 1985) or a primary amino acid motif (Rothbard and Taylor, 1988). Further, the conformation of an antigenic determinant is dependent not only on the residues making up the determinant but also on surrounding residues (Novotny, 1990). In theory, a chimaeric protein to be used for antigen presentation should include residues adjacent to the antigenic determinant and nearby cysteine residues involved in disulphide bond formation in an effort to ensure minimal alterations to the final protein conformation. In order for chimaeric rabies/VSV glycoproteins, such as those described in CHAPTER 4, to induce neutralizing responses against both VSV and rabies viruses, the

conformation of antigenic determinants present on the chimaeric protein must resemble the conformation of antigenic determinants on the parental glycoproteins. While subunit vaccines are based on the exact identification of the residues located in an epitope, a chimaeric glycoprotein would not require a precise definition of the epitopes but would include adjacent residues that affect presentation of the determinant to the immune system and other residues which may or may not contribute to the overall conformation.

Two types of chimaeric proteins can be constructed: chimaeric proteins containing direct domain replacements (*i.e.* ectodomain or cytoplasmic domain) or sequence replacements within a domain. Viral membrane protein domains are independently folding units, and the replacement of a complete domain is tolerated (reviewed in Doms *et al.*, 1993). Chimaeric proteins with near exact domain exchanges between VSV G and other viral membrane proteins are expressed on the cell surface (McQueen *et al.*, 1986; Puddington *et al.*, 1986; Roth *et al.*, 1986; Lasarovits *et al.*, 1990; Whitt *et al.*, 1991). A substitution of the influenza virus hemagglutinin (HA) cytoplasmic domain with the VSV G cytoplasmic domain (HA-HA-G) generates a trimerized molecule that is transported to the cell surface (Lasarovits *et al.*, 1990; Thomas *et al.*, 1993). Proteins containing substitutions of the HA transmembrane domain with the corresponding VSV G sequences (HA-G-G, HA-G-HA) are not assembled into trimers although oligosaccharide groups are added and correctly processed (McQueen *et al.*, 1986; Roth *et al.*, 1986; Lasarovits *et al.*, 1990). Immunological differences are detected on the ectodomain of HA-G-G when compared to wild type HA (Roth *et al.*, 1986). A chimaeric rabies/VSV glycoprotein, containing the rabies G ectodomain and VSV G transmembrane and cytoplasmic domains, is assembled into trimers, N-glycosylated, processed, and transported with kinetics similar to rabies glycoprotein but does not exhibit fusion activity which may be due to minor misfolding or an inability to undergo necessary conformational changes (Whitt *et al.*, 1991).

Successful sequence replacements within a domain are difficult to predict, and the generated proteins are typically misfolded and not assembled into oligomers (Kreis and Lodish, 1986; Lasarovits *et al.*, 1990; Doms *et al.*, 1993). Proteins GHA (VSV G aa 1-73/influenza HA aa 57 - carboxy terminus) and HAG (HA aa 1-422/VSV G aa 262-512) are glycosylated in the endoplasmic reticulum (ER) but are not transported to the Golgi for further processing (McQueen *et al.*, 1984). Intradomain chimaeric proteins of VSV Indiana and NJ G, such as constructs

WK10 (Ind aa 1-43/NJ aa 43-301/Ind aa 290-511), WK11 (Ind aa 1-43/NJ aa 43-298/Ind aa 317-511), WK12 (Ind aa 1-43/NJ aa 43-298), WK13 (Ind aa 1-43/NJ aa 43-267/Ind aa 317-511), WK14 (Ind aa 1-200/NJ aa 214-517), WK15 (Ind aa 1-297/NJ aa 294-517), and WK16 (Ind aa 1-201/NJ aa 321-517), migrated as glycosylated species in SDS-PAGE, but, unfortunately, were not examined for correct protein folding, assembly, oligosaccharide processing, and cell surface expression (Keil and Wagner, 1989). Chimaeric proteins containing amino terminal rabies (SAD) glycoprotein and carboxy terminal Mokola (a rabies-related virus) glycoprotein sequences and vice versa, or a SAD glycoprotein with a replacement of antigenic site III with the corresponding sequences from Mokola were glycosylated similar to the wild type proteins, detected at the surface of infected BSR cells, and recognized by rabies- or Mokola-specific antibodies (Mebatsion and Conzelmann, 1993).

I constructed and detected expression of five chimaeric rhabdoviral glycoproteins containing both rabies and VSV sequences in the ectodomain (RV1, VR1, VR2, VR3, and VR4). No published intradomain chimaerics correspond exactly to those generated in this work although HAG (McQueen *et al.*, 1984) and WK10, WK11, and WK13 (Keil and Wagner, 1989) contain most of the VSV Ind ectodomain sequences that are present in RV1 in this work, and WK14 and WK16 (Keil and Wagner, 1989) contain the same portion of VSV Indiana G ectodomain sequences as does VR3 in this work. Rabbit anti-rabies polyclonal antiserum precipitated the five proteins from radiolabelled, infected cell extracts; this indicates that the anti-rabies antiserum contains at least one population of antibodies specific for an antigenic determinant on the chimaeric glycoprotein and that the conformation of the determinant resembles a determinant of the parent protein. A second explanation may be that the anti-rabies antiserum contains a subpopulation of antibodies specific for a linear neutralizing epitope, G5, consisting of residues 279 to 286 of the rabies glycoprotein (van der Heijden *et al.*, 1993) and common to constructs VR1, VR2, VR3, and VR4. Initially, rabbit anti-VSV Indiana antiserum failed to precipitate RV1 (Fig. 4.3) while in a later experiment evaluating chimaeric protein glycosylation I observed that a different preparation of anti-VSV Indiana glycoprotein polyclonal antiserum was able to precipitate RV1. The failure of anti-VSV antiserum to detect RV1 in the initial expression studies suggests that antibodies reactive with the VSV G sequences in the RV1 protein were absent from the rabbit anti-VSV Ind antiserum. A conformational difference in the VSV G sequences present

in RV1 compared to the parental VSV G can be evaluated experimentally. Monoclonal antibodies, previously used to map VSV Ind epitopes A1 and B2 (Lefrançois and Lyles, 1982a, 1982b, 1983a) which are present in the RV1 sequence, would indicate if the conformation of the VSV portion of the RV1 ectodomain resembles that of the parent glycoprotein. Alternatively, chimaeric VSV NJ/Ind proteins, such as those described by Keil and Wagner (1989), can be precipitated with the anti-VSV Indiana polyserum used in this work. This approach may not be useful if the polyserum detects amino-terminal epitopes shared by Ind and NJ glycoproteins.

Higher levels of VR proteins were precipitated with anti-VSV polyserum than with anti-rabies antibodies (Fig. 4.3) which may indicate a stronger antibody affinity towards the VSV portion of the ectodomain or inaccuracies in the volume of cell extract. With the exception of RV1, the molecular weight (M_w) of the chimaeric proteins was higher than the predicted M_w (Table 4.1). Although VSV Ind G contains two 3.4 kD oligosaccharide side chains (Etchison and Holland, 1974), its apparent mobility and that of rabies G increase by 10 kD during SDS-PAGE (Knipe *et al.*, 1975; this work). SDS binds hydrophobic regions of proteins at a constant weight ratio (1.4 g SDS/g protein) resulting in protein separation based on polypeptide size (See and Jackowski, 1989). SDS does not bind carbohydrate groups resulting in a reduction of the net protein charge and lower protein mobility, especially in glycoproteins with more than 10% carbohydrate (See and Jackowski, 1989; Hames, 1990). It is predicted that oligosaccharides make up 15-17% of the total glycoprotein weight of VR1, VR2, VR3, and VR4 which accounts for a higher M_w for the recombinant proteins than would be calculated.

The number of N-glycosylation sites in the chimaeric proteins differs from the number of groups present in the parent proteins. RV1 contains one potential glycosylation site while the VR constructs contain three potential glycosylation sites. Removal or addition of glycosylation consensus sequences is known to lead to defects at the folding stage; in the absence of glycosylation VSV and rabies G are detected in the ER as aggregates of aberrantly folded and disulphide-bonded protein monomers that fail to be transported to the cell surface (Gibson *et al.*, 1979; Machamer and Rose, 1988a, 1988b; Machamer *et al.*, 1990; Burger *et al.*, 1991) and reduced reactivity with MAbs is observed (Keil and Wagner, 1989). The addition of glycosylation sites to other regions of VSV or rabies G does not correct the inaccurate post-translational processing and aggregation (Doms *et al.*, 1988; Machamer *et al.*, 1990) and results

in the loss of antigenic determinants (Wunner *et al.*, 1985; Machamer *et al.*, 1990). Infrequently, additional glycosylation sites are tolerated although the intracellular rate of transport is decreased (Machamer and Rose, 1988b). Thus, there is a good probability that the alteration to the total number of oligosaccharide groups has had a negative impact on chimaeric protein processing and transport through the ER and Golgi pathways.

β -endo-*N*-acetyl glucosaminidase H (Endo H), specific for N-linked high mannose sugars, was observed to cleave the oligosaccharide groups on protein chimaeras RV1, VR1, and VR3. Typically, rabies glycoprotein acquires Endo H resistance with a $t_{1/2}$ of 50 min (Whitt *et al.*, 1991), whereas VSV glycoprotein is folded at a $t_{1/2}$ of 7-8 min and becomes Endo H resistant at a $t_{1/2}$ of 15-20 min (Rose and Bergmann, 1983). In my work chimaeric proteins VR1 and VR3, after a pulse-label for two hours, remained Endo H sensitive (Fig. 4.5). This would indicate that the carbohydrate groups are not processed in a post-ER compartment and that the chimaeric proteins are not transported to the medial Golgi (Beeley, 1985). An alternate explanation would be that the $t_{1/2}$ for protein folding exceeds two hours. As $t_{1/2}$ is an average folding time, there should exist a small fraction of proteins with a folding time less than two hours and with a higher probability of detection in a processed and Endo H resistant state.

The chimaeric molecules constructed in this work contain intradomain replacements which effectively remove or delete certain sequences. It is known that some VSV G ectodomain deletion mutant proteins remained in the ER in an unstable association with GRP78-BiP, a resident ER protein involved in protein folding and the prevention of protein aggregation (DeSilva *et al.*, 1990; Machamer *et al.*, 1990; Doms *et al.*, 1993). Intrachain disulphide bonds in ectodomains are required for protein folding, maturation, and stability, and the deletion or addition of cysteine residues leads to protein misfolding and aggregation (Doms *et al.*, 1993). The removal of one cysteine residue from a binding pair of cysteines in HSV glycoprotein D (gD) causes a higher degree of misfolding as the remaining cysteine residue has the option of interacting with any other cysteine residue in the protein sequence (Wilcox *et al.*, 1988; Long *et al.*, 1992). Native gD conformation is maintained with the removal of a matched pair of cysteines (Long *et al.*, 1992). All cysteine residues are conserved in VSV Ind and NJ glycoproteins, while nine out of twelve cysteines are in homologous positions of Indiana and rabies glycoproteins (Grigera *et al.*, 1992). Two major disulphide loops are predicted to

commence within the first 193 residues of the VSV NJ glycoprotein (Cys 108 binding Cys 169, and Cys 130 binding to Cys 235, Cys 240, or Cys 273) and influence the recognition of epitopes distal to residue 193 (Grigera *et al.*, 1992). If this disulphide binding pattern is applicable to VSV Indiana G, then all VR constructs described in this work could potentially form a Cys 108-Cys 169 bond, while only VR1 and VR2 would have the potential for the second disulphide loop. Grigera and researchers (1992) have not determined minor disulphide bonding for VSV G and, indeed, the disulphide bonding pattern of rabies glycoprotein remains to be determined. It is possible that the conformations of the VR and RV chimaeric proteins are altered due to aberrant disulphide bonds and this has led to protein aggregation in the ER or post-ER prior to the medial Golgi.

FAILURE TO DETECT VIRUS NEUTRALIZING ANTIBODY TO CHIMAERIC RHABDOVIRAL GLYCOPROTEINS IN MICE

The practicality and effectiveness of chimaeric rhabdoviral glycoproteins in an immunization strategy was tested in a mouse model. Mouse cells, although only semi-permissive for human adenovirus replication (Martins, 1991), have proven a useful model for examining foreign protein expression *in vivo* and in determining the potential to evoke an immune response. Previously constructed vectors containing sequences for either of the parental glycoproteins, rabies (ERA) or VSV (Indiana), were repeatedly shown to induce good levels of virus neutralizing antibodies (Schneider, 1989; Schneider *et al.*, 1989; Prevec *et al.*, 1990; R. Spessot and L. Prevec, personal communication; this work, CHAPTER 3). HAd5 vectors containing chimaeric rhabdoviral glycoprotein sequences were unable to generate a neutralizing antibody response against the parental glycoproteins. If the glycoprotein chimaera was in contact with the immune system, why, then, would no antibody be detected? It is possible that, if the chimaeric glycoprotein is expressed and antibodies are generated, then there is no subpopulation of antibody able to recognize the parental virus. An argument against this possibility is that I had used polyserum directed against the parental glycoproteins to detect the chimaeric proteins in infected cultured cell extracts. The positively reacting antibody subpopulation, though, may be directed against non-neutralizing epitopes. I might have addressed this issue by attempting to

immunoprecipitate chimaeric proteins VR1, VR2, VR3, and VR4 with a monoclonal specific for a linear neutralizing epitope present on the rabies glycoprotein (van der Heijden *et al.*, 1993). A positive reaction would indicate that the VR proteins have the potential to contain neutralizable epitopes. To further determine if the chimaeric proteins were immunogenic and able to elicit an antibody response, immune serum from rabbits immunized with the chimaeric glycoprotein-expressing vectors could be assessed for its ability to precipitate the chimaeric proteins from infected cells. The development of a neutralizing antibody response could be measured by testing serum generated against the purified chimaeric glycoproteins for neutralization with the parental VSV or rabies viruses.

Previous studies with AdRG1 had shown that some mice immunized with the recombinant virus generated low or negative levels of rabies virus neutralizing antibodies yet remained protected from disease development upon challenge with live rabies virus (Prevec *et al.*, 1990). This observation supported previous work that a cell-mediated response against the glycoprotein is required to protect against rabies infection (Wiktor *et al.*, 1977; Wiktor, 1978; Wiktor *et al.*, 1984). We know that construct RV1 contains a region deemed important for recognition by MHC class I-restricted CTL (Macfarlan *et al.*, 1986). Are chimaeric glycoproteins RV1, VR1, VR2, VR3, and VR4 degraded and presented by MHC to be recognized by cytotoxic T lymphocytes? Potentially, mice immunized with the chimaeric vectors could be challenged with rabies or VSV virus and observed for survival and recovery. Alternatively, lymphocytes from mice immunized with the chimaeric glycoprotein vectors could be evaluated for cytotoxicity against a target population of antigen presenting cells infected with wild type VSV or rabies virus, or with vaccinia virus vectors expressing rabies glycoprotein. These experiments, if carried out with AdRV1, would indicate if the rabies glycoprotein CTL domain is processed and presented, and, using all five chimaeric glycoprotein vectors, might be informative as to the location of the VSV glycoprotein CTL domain.

FUTURE PROSPECTS OF CHIMAERIC RHABDOVIRAL GLYCOPROTEINS

Several questions have arisen at the completion of this research. What is the fate of the chimaeric molecules? Is there a structural resemblance between the chimaeric molecules and the

parental glycoproteins? How can the chimaeric glycoproteins be constructed to maintain the conformation of the parental glycoproteins? Clearly, without a detailed analysis of the crystal structure of these molecules it is difficult to address these issues. The use of sequence similarities and evolutionary similarities did not facilitate my attempt to construct chimaeric rhabdoviral glycoproteins. One solution may be to take the approach pursued by Mebatsion and Conzelmann (1993): to substitute a defined epitope on the rabies glycoprotein with a defined epitope from VSV glycoprotein. This small substitution might be less harmful to the overall structure of the protein with less impact on protein folding, assembly, and intracellular transport than the approach pursued in this study. With the current problems of constructing subunit vaccines composed of the correct B and T cell determinants and obtaining good immune responses, and a questionable efficacy of attenuated or killed, whole rabies virus vaccines in mediating protection against disease development in a wide host range, the generation of chimaeric protein molecules should continue to be pursued.

APPENDIX 1

RABIES VIRUS NEUTRALIZING ANTIBODY TITERS OF SKUNKS
IMMUNIZED WITH RECOMBINANT Ad/RABIES G VIRUS

Virus	Titer	Animal#	VNA Titer (International Units)					
			Pre	14	30	60	90	120 days
Study #1 (ADRI 74)								
AdRG1	2 x 10 ⁷ (10 ^{7.3})	1474R	0.0	2.78	11.1	22.3	11.1	11.1
		1477R	0.0	0.0	0.7	0.7	0.35	0.35
		1478R	0.0	0.0	0.0	0.17	0.0	0.17
		1479R	0.0	0.0	0.17	0.35	0.35	0.7
		1481R	0.0	0.0	0.0	0.0	0.0	0.0
		1482R	0.0	5.57	11.1	44.5	5.57	11.1
		1483R	0.0	0.0	0.0	0.7	0.35	0.35
		1484R	0.0	0.7	5.57	11.1	2.78	5.57
		Mean	0.0	1.13	3.6	10.0	2.6	3.7
		SD	0.0	2.0	5.0	16.1	4.0	4.9
AdRG1	2 x 10 ⁶ (10 ^{6.3})	1473R	0.0	0.0	0.0	0.7	0.7	2.78
		1475R	0.0	0.0	0.0	0.0	0.0	0.0
		1476R	0.0	1.39	44.5	44.5	44.5	44.5
		1480R	0.0	0.0	0.0	0.0	0.35	0.0
		1492R	0.0	0.0	0.0	0.0	1.39	0.0
		1493R	0.0	0.0	5.57	44.5	22.3	22.3
		1494R	0.0	0.7	5.57	11.1	2.78	5.57
		1495R	0.0	0.0	0.0	0.35	0.35	0.35
		Mean	0.0	0.26	7.0	12.6	9.0	9.4
		SD	0.0	0.5	15.4	20.0	16.2	16.1
AdRG1	2 x 10 ⁵ (10 ^{5.3})	1485R	0.0	0.0	0.0	0.0	0.0	0.0
		1486R	0.0	0.0	0.0	0.0	0.0	0.0
		1487R	0.0	0.0	0.0	0.0	1.39	0.0
		1488R	0.0	0.0	0.0	0.0	0.35	0.0
		1489R	0.0	0.0	0.0	0.0	0.0	0.0
		1490R	0.0	0.0	0.0	0.0	0.0	0.0
		1491R	0.0	0.0	0.0	0.0	0.0	0.0
		1496R	0.0	0.0	0.0	0.0	0.0	0.0
		Mean	0.0	0.0	0.0	0.0	0.2	0.0
		SD	0.0	0.0	0.0	0.0	0.5	0.0

Virus	Titer	Animal#	VNA Titer (International Units)					
			Pre	14	30	60	90	120 days
AdRG4	2 x 10 ⁷ (10 ^{7.3})	1502R	0.0	0.0	44.5	44.5	11.1	11.1
		1504R	0.0	2.78	44.5	89.0	44.5	22.3
		1506R	0.0	5.57	89.0	89.0	22.3	11.1
		1508R	0.0	2.78	11.1	22.3	5.57	22.3
		1513R	0.0	0.35	5.57	11.1	5.57	5.57
		1515R	0.0	1.39	11.1	89.0	89.0	89.0
		1517R	0.0	0.0	0.35	0.35	0.7	0.7
		1519R	0.0	0.0	0.0	0.0	0.0	0.0
		Mean	0.0	1.6	25.8	43.2	22.3	20.3
		SD	0.0	2.0	31.3	40.5	30.7	29.1
AdRG4	2 x 10 ⁶ (10 ^{6.3})	1498R	0.0	0.0	0.35	0.7	0.35	0.7
		1500R	0.0	0.0	0.35	0.7	0.35	0.35
		1505R	0.0	0.0	0.17	0.7	0.7	0.17
		1507R	0.0	0.35	5.57	5.57	2.78	5.57
		1509R	0.0	0.17	0.7	1.39	0.35	1.39
		1511R	0.0	0.35	2.78	2.78	1.39	0.7
		1518R	0.0	0.0	0.17	0.7	0.7	0.35
		1520R	0.0	0.35	11.1	5.57	11.1	11.1
		Mean	0.0	0.15	2.6	2.3	2.2	2.5
		SD	0.0	0.2	3.9	2.2	3.7	3.9
AdRG4	2 x 10 ⁵ (10 ^{5.3})	1497R	0.0	0.0	0.0	0.0	0.0	0.0
		1499R	0.0	0.0	0.0	0.0	0.0	0.0
		1501R	0.0	0.0	0.0	0.0	0.0	0.0
		1503R	0.0	0.0	0.0	0.0	0.0	0.0
		1510R	0.0	0.0	0.35	0.17	0.35	0.0
		1512R	0.0	0.0	0.0	0.0	0.0	0.0
		1514R	0.0	0.0	0.0	0.0	0.0	0.0
		1516R	0.0	0.0	1.39	0.35	0.7	0.0
		Mean	0.0	0.0	0.22	0.07	0.13	0.0
		SD	0.0	0.0	0.5	0.13	0.3	0.0

Study #2 (ADRI 86)

AdRG1.3	3.2 x 10 ⁷	1609R	0.0	22.3	11.1	11.1
		1646R	0.0	89.0	17.8	89.0
		1690R	0.0	89.0	44.5	44.5
		1693R	0.0	0.0	0.0	0.0
		Mean	0.0	50.1	58.4	36.2
		SD	0.0	45.9	82.0	40.0

Virus	Titer	Animal#	VNA Titer (International Units)				
			Pre	14	30	60	90
AdRG1.3	3.2 x 10 ⁶	1621R	0.0	44.5	44.5	178.0	
		1647R	0.0	22.3	89.0	44.5	
		1691R	0.0	0.0	0.35	1.39	
		1692R	0.0	89.0	4.5	89.0	
		Mean	0.0	39.0	44.6	78.2	
		SD	0.0	38.0	32.2	75.5	
Study #3 (ADRI 87)							
AdRG1	1.1 x 10 ⁷	1721R	0.0	22.3	44.5	89.0	89.0
		1722R	0.0	0.35	2.78	11.1	5.57
		1723R	0.0	89.0	178.0	89.0	44.5
		1724R	0.0	0.7	1.39	2.78	0.35
		1726R	0.0	0.0	0.0	0.0	0.17
		1741R	0.0	11.1	44.5	11.1	5.57
		1746R	0.0	0.35	22.3	44.5	22.3
		1747R	0.0	2.78	22.3	44.5	11.1
		Mean	0.0	15.8	39.5	36.5	22.32
		SD	0.0	30.6	58.8	36.6	
AdRG1	6.4 x 10 ⁵	1730R	0.0	0.17	11.1	44.5	11.1
		1732R	0.0	5.57	89.0	44.5	no sample
		1734R	0.0	0.0	0.7	2.78	1.39
		1736R	0.0	0.0	2.78	11.1	2.78
		1737R	0.0	0.0	0.0	0.17	0.0
		1739R	0.0	0.0	5.57	5.57	2.78
		1743R	0.0	0.0	0.7	1.39	0.7
		1745R	0.0	1.39	44.5	44.5	22.3
		Mean	0.0	0.9	19.3	19.3	5.86
		SD	0.0	2.0	31.8	21.1	
AdRG1	2.2 x 10 ⁵	1727R	0.0	0.0	0.0	0.0	0.0
		1728R	0.0	0.0	0.0	0.0	0.0
		1729R	0.0	0.0	0.0	0.0	0.0
		1731R	0.0	0.0	5.57	11.1	5.57
		1735R	0.0	0.0	0.0	0.0	0.17
		1738R	0.0	0.0	0.0	0.17	0.35
		1748R	0.0	0.0	0.0	0.7	0.35
		1750R	0.0	0.0	0.0	0.0	0.0
		Mean	0.0	0.0	0.7	2.9	0.81
		SD	0.0	0.0	2.0	7.8	

Virus	Titer	Animal#	VNA Titer (International Units)					120 days
			Pre	14	30	60	90	
AdRG1.3	2×10^7	1663R	0.0	11.1	178.0	89.0	22.3	
		1665R	0.0	89.0	178.0	178.0	44.5	
		1674R	0.2	0.17	178.0	178.0	44.5	
		1678R	0.0	0.0	0.0	0.0	0.35	
		1679R	0.0	89.0	89.0	89.0	22.3	
		1680R	0.0	11.1	89.0	89.0	44.5	
		1683R	0.0	89.0	178.0	178.0	178.0	
		1688R	0.0	44.5	178.0	89.0	22.3	
		Mean	0.0	41.7	133.5	111.3	47.34	
		SD	0.1	41.5	67.3	62.9		
AdRG1.3	3.6×10^5	1659R	0.0	2.78	5.57	89.0	44.5	
		1664R	0.0	1.39	22.3	44.5	44.5	
		1667R	0.0	44.5	89.0	175.0	44.5	
		1668R	0.0	2.78	22.3	89.0	44.5	
		1670R	0.0	2.78	22.3	89.0	0.7	
		1681R	0.0	0.17	11.1	11.1	5.57	
		1685R	0.0	0.35	1.39	5.57	5.57	
		1694R	0.0	2.78	5.57	11.1	5.57	
		Mean	0.0	7.2	19.8	53.5	24.43	
		SD	0.0	15.1	29.2	60.7		
AdRG1.3	6×10^4	1658R	0.0	0.0	1.39	5.57	22.3	
		1660R	0.0	0.0	0.17	0.35	1.39	
		1662R	0.0	0.0	2.78	2.78	1.39	
		1666R	0.0	0.0	0.7	1.39	0.7	
		1669R	0.0	0.0	0.0	0.0	0.0	
		1671R	0.0	0.0	2.78	2.78	0.7	
		1675R	0.0	0.17	1.39	2.78	2.78	
		1677R	0.0	0.0	0.17	0.7	2.78	
		Mean	0.0	0.0	1.2	2.0	4.01	
		SD	0.0	0.1	1.1	1.8		

Virus	Titer	Animal#	VNA Titer (International Units)					
			Pre	14	30	60	90	120 days
Study #4 (ADRI 93)								
ADHCMV13RG								
	5.6 x10 ¹⁰	1649R	0.0	22.3	44.5	11.1	5.57	2.78
		1653R	0.0	11.1	89.0	22.3	22.3	22.3
		1760R	0.0	2.78	44.5	22.3	22.3	11.1
		Mean	0.0	12.1	59.3	18.6	16.72	12.06
		SD	0.0	9.8	25.7	6.5		
	1.8 x10 ⁸	1651R	0.0	0.0	11.1	11.1	22.3	11.1
		1655R	0.0	2.78	44.5	44.5	22.3	22.3
		1759R	0.0	2.78	5.57	11.1	5.57	5.57
		Mean	0.0	1.9	20.4	22.2	16.72	12.99
		SD	0.0	1.6	21.1	19.3		

International Units are calculated from FIMT Equivalent Units by dividing by 46. FIMT EU are based on an ELISA assay specific for rabies glycoprotein.

APPENDIX 2

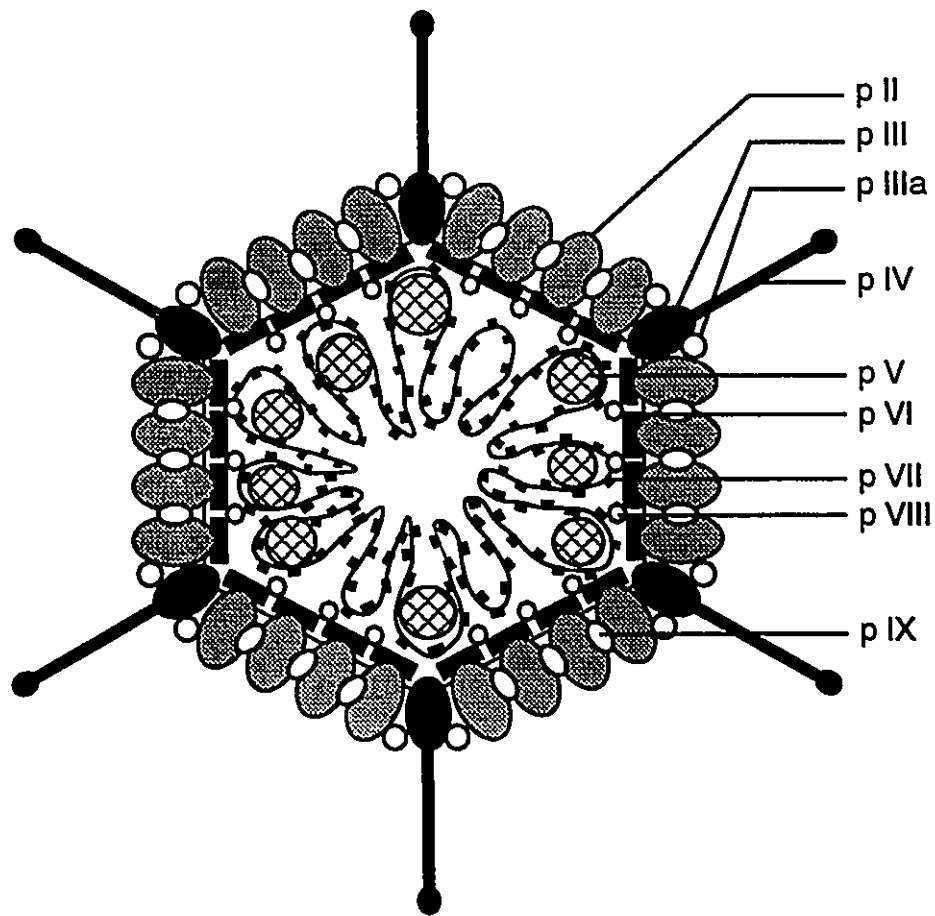
CHARACTERIZATION OF MONOCLONAL ANTIBODIES DIRECTED AGAINST ADENOVIRUS

The production of monoclonal antibodies (MAbs) against adenoviral proteins has aided the study of viral structure and protein antigenicity. MAbs have enabled studies of the virus replication cycle in cells that are permissive, semi-permissive, or non-permissive for adenovirus replication (Martins, 1991). Further uses of anti-adenoviral MAbs include the determination of virus pathogenicity in animal models immunized with recombinant adenovirus-based vaccines.

Adenoviruses consist of an outer protein coat /capsid/ and a core component containing the viral DNA, as well as capsid-associated and DNA-associated proteins (reviewed in Nermut, 1984; Pettersson, 1984). The outer capsid is comprised of three proteins: hexon, penton base, and fiber, with the latter two proteins forming the penton capsomere (Fig. 6.1). Hexon, also known as protein II, localizes to the triangular faces of the virion. Penton base and fiber, proteins III and IV respectively, localize to the twelve vertices of the icosahedral structure. In total, the viral capsid contains 240 hexon and 12 penton macromolecules. Capsid proteins are the primary antigens recognized by the immune system. Sequence diversity of hexon, penton base, and fiber antigenic determinants are used as criteria in the classification of adenovirus serotypes.

HEXON

The hexon protein plays a structural role in the adenoviral virion. Between Ad serotypes there exists minimal sequence variation for hexon with high sequence conservation at the ends of the molecule and clusters of variation between serotypes localizing to the amino terminal half of the protein (Roberts *et al.*, 1986). Ad2 and Ad5 share identity over 88% of the amino acid sequence (Kinloch *et al.*, 1984). Restriction fragment comparison of Ad2 and Ad5 hexons indicates the presence of heterologous sites between residues 140 and 290 in the amino-terminal



Designation	Protein	MW
p II	hexon	120 kD
p III	penton base	85 kD
p IIIa	penton base-associated	66 kD
p IV	fiber	62 kD
p V	core protein	48.5 kD
p VI	hexon-associated	24 kD
p VII	core protein	18.5 kD
p VIII	hexon-associated	13 kD
p IX	hexon-associated	12 kD

FIGURE 6.1: REPRESENTATION OF AN ADENOVIRUS

Modified from Horwitz, 1990.

half of the protein - this region of the Ad2 protein contains trypsin-sensitive sites (positions 142-143, 165-166, and 286-287) and is predicted to contain type-specific antigenic determinants of the exposed surface (Boursnell and Mautner, 1981; Von Bahr-Lindström *et al.*, 1982).

Initial studies demonstrated that adenoviruses shared a cross-reacting group antigen that mapped to the inner surface of the hexon capsomere (Pereira *et al.*, 1959; Wilcox and Ginsberg, 1963a, 1963b; Wilcox *et al.*, 1963; Valentine and Pereira, 1965; Norrby *et al.*, 1969), as well as a type-specific determinant found on the outer surface (Wilcox and Ginsberg, 1963a, 1963b; Kasel *et al.*, 1964, 1966; Kjellen and Pereira, 1968; Norrby *et al.*, 1969; Willcox and Mautner, 1976a, 1976b). To date, the complexity of the determinants present on the hexon protein indicates the existence of type, group, intrasubgroup, and intersubgroup specificities (Norrby and Wadell, 1969b; Russell *et al.*, 1981; Pettersson, 1984).

Evidence supporting the presence of a neutralizing type-specific antigenic determinant on hexon has come from numerous groups (Wilcox and Ginsberg, 1963a, 1963b; Kjellen and Pereira, 1968; Pereira and Laver, 1970; Haase and Pereira, 1972; Wadell, 1972; Willcox and Mautner, 1976a, 1976b; Wöhlhart *et al.*, 1985; Wöhlhart, 1988), while other groups have failed to detect neutralizing activity (Pettersson *et al.*, 1967; Norrby, 1969a, 1969b). Pereira and Laver (1970) in immunodiffusion studies using monospecific serum against the purified protein showed a cross-reactivity between Ad2 and Ad5 hexons but found virus neutralization to be strictly type-specific. During a primary immune response 0.1-1% of the antibody produced was neutralizing type-specific for hexon, while during a secondary response the level of this specific antibody rose to 10-20% suggesting that type- and group-specific antibodies directed against hexon are separate populations (Willcox and Mautner, 1976a, 1976b). In pH-dependent cleavage studies of the Ad2 hexon, Varga *et al.* (1990) detected the production of a 15 kD hexon fragment at pH 5-6 corresponding to the first 140-160 residues of the amino terminus. Antibodies raised against the 15 kD fragment demonstrated a five fold higher pH-independent neutralizing capacity than polyclonal anti-hexon serum (Varga *et al.*, 1990). The residue area of 15 kD maps to the area of high sequence diversity observed by Roberts *et al.* (1986) and corresponds to the region of hexon containing type-specific neutralizing determinants.

PENTON

The penton capsomere consists of penton bases and protruding fiber molecules which interact in a noncovalent manner.

Fiber

The primary role of fiber is the recognition of receptors on the plasma membrane during the first stages of adenoviral infection (Levine and Ginsberg, 1967; Philipson *et al.*, 1968; Londberg-Holm and Philipson, 1969). When added to cells, purified fiber can inhibit virus attachment to the receptor (Philipson *et al.*, 1968), while *in vitro* studies have shown the ability of fiber to bind DNA thus inhibiting the action of DNA and RNA polymerases (Pettersson, 1984). The fiber molecule consists of an amino-terminus shaft and a carboxy-terminus knob component with the shaft portion interacting with penton base (Devaux *et al.*, 1987). The shaft is not highly conserved between serotypes but the periodicity of proline and hydrophobic residues between amino acids 50 and 400 is maintained (Green *et al.*, 1983; Chroboczek and Jacrot, 1987). Ad2 and Ad5 fiber molecules share 69% homology and have an identical sequence for the first fifty residues (Chroboczek and Jacrot, 1987). The fiber protein contains O-linked N-acetylglucosamine groups buried within the trimeric structure; these may be important for fiber stabilization (Mullis *et al.*, 1990) and were shown to be required for an immune response (Chee-Sheung and Ginsberg, 1982).

The fiber protein carries type-, group-, or subgroup-specific determinants. Type-specific determinants map to the carboxy-terminus knob of fiber (Valentine and Pereira, 1965; Norrby, 1966b, 1969a; Pettersson *et al.*, 1968). The type-specific determinant can generate both neutralizing (Wilcox and Ginsberg, 1963b; Valentine and Pereira, 1965; Norrby, 1966b, 1968; Wadell and Norrby, 1969; Wöhlhart *et al.*, 1985; Wöhlhart, 1988) and non-neutralizing antibodies (Kjellen and Pereira, 1968; Pettersson *et al.*, 1968; Haase and Pereira, 1972; Watson *et al.*, 1988); these conflicting observations vary according to laboratory methodologies. Immunofluorescent labelling has detected anti-fiber Ab neutralization of Ad infection (Philipson,

1961), but no virus neutralization is observed in plaque assays with anti-fiber polyserum (Philipson, 1961; Haase and Pereira, 1972). Watson *et al.* (1988), employing other techniques, have described five epitopes mapping to the knob structure with four of the five epitopes generating neutralizing antibodies. Anti-fiber serum generated during an immune response contains a neutralizing capacity approaching that obtained with anti-adenovirus polyserum (Wöhlhart *et al.*, 1985; Wöhlhart, 1988). Adenovirus neutralization using anti-fiber antibodies is extracellular occurring at the level of virion adsorption to the cell (Wöhlhart, 1988). The subgroup determinant of fiber localizes to the junction near penton base (Pettersson and Höglund, 1969; Wadell and Norrby, 1969; Boudin and Boulanger, 1981). Although most work indicates that the subgroup determinant is non-neutralizing, Watson *et al.* (1988) characterized several MAbs specific for subgroup determinants that were able to generate a neutralizing response. Intersubgroup determinants of the fiber molecule have also been characterized for adenoviruses of subgenera C and D (Pettersson *et al.*, 1968; Norrby, 1968, 1969a).

Penton Base

In addition to a structural role in the virion, purified penton base is toxic to cells inducing an early cytopathic effect (CPE) (Valentine and Pereira, 1965; Pettersson and Höglund, 1969). The toxicity is reversible by changing the media, or by treating penton base with anti-penton sera or low amounts of trypsin (Pettersson and Höglund, 1969; Wadell and Norrby, 1969). A cell-derived endonuclease activity specific for ssDNA or GC regions of dsDNA associates with pentons in Ad2- or Ad12-infected cells (Burlingham *et al.*, 1971; Marusyk *et al.*, 1975). Penton base may also be required for adenovirus movement from endosomal compartments into the cytoplasm (Svensson, 1985).

Penton base contains a weak determinant common to all pentons, as well as intergroup and intrasubgroup determinants (Pereira and de Figueiredo, 1962; Valentine and Pereira, 1965; Norrby, 1966b; Norrby and Wadell, 1967; Kjéllen and Pereira, 1968; Wadell and Norrby, 1969). Kjéllen and Pereira (1968) were able to raise anti-penton base polyserum with low levels of neutralizing activity. Anti-penton base polyserum fails to inhibit Ad2 attachment and penetration of HeLa cells but can neutralize 45% of virus at serum dilutions of 1/1.1 and 1/1.25 when

reacted at a neutral pH (Wöhlhart *et al.*, 1985; Wöhlhart, 1988). Complete neutralization is obtained with virus reacted with anti-penton antibody at pH 5.0 (Wöhlhart, 1988). By partitioning ³⁵S-labelled proteins between an aqueous phase and a detergent phase at decreasing pH, Wöhlhart observed an increase in the hydrophobicity of penton base. The lower pH would expose unaccessible antigenic sites on the penton base thus permitting full neutralization. The significant amounts of virus present in the cytoplasm after anti-penton neutralization support an alternative mechanism of viral entry into cells, probably by direct penetration and not requiring penton base (Svensson, 1985).

OTHER ADENOVIRAL PROTEINS

Additional proteins localizing to the virion include polypeptides IIIa (66 kD), IV (62 kD), IVa₂ (50 kD), V (48 kD), VI (24 kD), VII (18.5 kD), VIII (15 kD), IX (14.3 kD), X (7 kD), XI (4.5 kD), and XII (3 kD), where polypeptide designation is based on relative mobility (refer to Figure 6-1). Basic polypeptides V and VII covalently associate with DNA (Russell *et al.*, 1971; Everitt *et al.*, 1973). pVII is considered a major core protein present at 1000 copies/virus particle, while there are 200 copies of pV per virus particle. Six pVII proteins associate with approximately 150 bp of DNA to form a nucleosome-like structure that is separated from the next nucleosome by a spacer region of pV attached to approximately 50 bp of DNA (Mirza and Werner, 1977, 1982). pV was localized adjacent to the vertices in close proximity to penton base, hexon, and pIIIa and may play a role in packaging virion DNA (Everitt *et al.*, 1975; Horwitz, 1990). pIIIa is located next to penton base; although detectable on the virion surface pIIIa likely extends through the capsid and serves as a bridge between peripentonal hexon and pVII (Everitt *et al.*, 1973, 1975; Pettersson, 1984; Horwitz, 1990). pIIIa is required in virion assembly as well as in the maintenance of capsid structure (Devaux *et al.*, 1982; Pettersson, 1984). pIVa₂ and VI are also capable of binding DNA (Russell and Precious, 1982). Hexons located at the triangular facets of the virion were associated with pVI, IX, and occasionally VIII, whereas hexon purified from infected cells could only associate with pII (Everitt *et al.*, 1973, 1975; Boulanger *et al.*, 1979; Horwitz, 1990). The functions of pIVa₂, pX, pXI, and pXII are not understood.

Due to the low levels present in infected cells, only a few of the core proteins have been examined for immunological properties. Everitt *et al.* (1975) and Boudin *et al.* (1980) have detected non-neutralizing group determinants on protein IIIa (located at the vertices at a copy number of five proteins per penton base). Polypeptide VII, a major core protein, is weakly immunogenic, and carries both group and type determinants (Prage and Pettersson, 1971). Ad2- and Ad5-derived polypeptide IX contains both type- and group-determinants; as well, treatment of viral particles with anti-protein IX antibodies results in particle aggregation suggesting an accessibility of determinants to the exterior of the particle (Maizel *et al.*, 1968; Everitt *et al.*, 1973; Boulanger *et al.*, 1979; Pettersson, 1984).

THE PROJECT

Panels of MAbs specific for human adenovirus (hAd) type 5 major structural proteins hexon, penton base, and fiber, as well as for protein IX and other core proteins have been reported (Cepko *et al.*, 1981; Russell *et al.*, 1981; Watson *et al.*, 1988; and others). As a result, type-, group-, intergroup-, and subgroup-specific determinants for the structural proteins were defined and the potential of individual polypeptides to elicit neutralizing antibodies was described. A panel of twelve monoclonal antibodies against adenovirus type 5 was prepared by Alex Wandeler (Animal Disease Research Institute, Agriculture Canada, Nepean, ON). This project involved the characterization of the MAb panel graciously provided by Dr. Wandeler. In this chapter I characterized twelve monoclonal antibodies specific for hAd5 structural proteins hexon, fiber, and protein VII.

MATERIALS AND METHODS

CELLS AND VIRUSES

HeLa (human aneuploid cervical carcinoma) cells were maintained as monolayers in α -minimal essential medium (α -MEM) supplemented with 10% (v/v) newborn calf serum,

penicillin-streptomycin (100 U/ml and 100 µg/ml, respectively), and 25 U/ml nystatin suspension (all obtained from Gibco BRL Life Technologies, Inc., Canada).

Virus stocks used in the study included human Adenovirus (hAd) types 2 and 5 and AdlacZ. AdlacZ is a recombinant Ad5 containing an *Xba*I deletion in the E3 region replaced with sequences for the SV40 early promoter and β-galactosidase (B. Christie and L. Prevec, personal communication). Human adenoviruses were propagated as described by Graham and Prevec (1991, 1992) and summarized in CHAPTER 2.

ANTIBODIES

A rabbit polyclonal serum raised against Ad5 was used to detect both Ad5 and Ad2 proteins (10 µl per reaction; G. Martins and L. Prevec, personal communication). Mouse monoclonal antibody H2-19 was specific for Ad5 72K protein (3-5 µl per reaction; Rowe *et al.*, 1984). Ad5 hexon protein was detected with monoclonal antibody (MAb) 9F6-6 (2 µl per reaction; J. Williams, University of Pittsburgh, USA), while Ad5 protein IX was detected with a monoclonal antibody raised against Ad2 protein IX (2 µl per reaction; W.C. Russell, University of St. Andrews, Scotland). Other monoclonal antibodies directed against Ad5 are characterized in this chapter.

IMMUNOPRECIPITATION OF VIRAL PROTEINS

HeLa cells were washed once with PBS⁺⁺ and infected with virus at a MOI of 20 pfu/cell. Virus was adsorbed for one hour at 37°C, and conditioned medium was added back to the cells. Cells were labelled for two hours with 250 µCi/150 mm dish of Tran^[35S]-label (ICN Biomedicals, Inc.) 18 hrs after infection. Medium was removed and cells were washed twice with PBS, followed by lysis with 8 ml of cold RIPA buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100). The extracts were kept at 4°C for 30 min and then passed through a 22G needle to shear the DNA.

In immunoprecipitations antibody was added to 300-500 µl of cell extract for one hour at 4°C; Protein A-Sepharose[®] CL4B (Pharmacia, Inc.) was added and incubation overnight at

4°C. Immunocomplexed beads were washed 3-5 times with cold RIPA buffer, resuspended in 50 μ l of 2X sample buffer (125 mM Tris, pH 7.2, 5% SDS, 12.5% β -mercaptoethanol, 25% glycerol, bromophenol blue), and boiled 5 min. Samples were analyzed on 9% or 10% discontinuous SDS-PAGE gels.

NEUTRALIZATION OF AdlacZ INFECTIVITY

HeLa cells were seeded in 24 well cell culture plates (Falcon) at a density of 2×10^5 cells per well. AdlacZ virus stock was diluted to 5×10^6 pfu/ml and 6×10^5 pfu were added to a serial dilution of antibody in PBS⁺⁺ (1/5, 1/25, 1/125, 1/625, 1/3125). Virus was neutralized for one hour at 37°C. HeLa cells were washed once with PBS⁺⁺, and 200 μ l of neutralized virus was allowed to adsorb for one hour at 37°C. Cells were washed once with prewarmed media to remove unadsorbed virus. Fresh media was added to the cells and cells were incubated for 23 hours at 37°C. Media was removed and cell extracts were assayed for β -galactosidase activity.

Cell extracts were prepared by resuspending the cells in 100 μ l of cold solution A (250 mM Tris, pH 7.8, 1 mM phenylmethylsulfonylfluoride, 0.5% NP40) and vortexing several times over 20 min. Solution B (875 μ l of 10 mM KCl, 1 mM MgSO₄, 100 mM sodium phosphate, 50 mM β -mercaptoethanol, pH 7.5) was added to each well, and plates were incubated at 37°C for 5 min. Solution C (330 μ l of *o*-nitrophenyl β -D-galactopyranoside (ONPG) (4 g/liter) in 100 mM sodium phosphate, pH 7.5) was added, and incubation continued at 37°C for one hour. Reactions were terminated with the addition of 430 μ l of 1 M Na₂CO₃. β -galactosidase activity was assayed by absorbance at 420 nm. The neutralizing dilution is the highest reciprocal serum dilution resulting in a 50% decrease in β -galactosidase activity.

RESULTS

MONOCLONAL ANTIBODY SPECIFICITY

To determine the specificity of each monoclonal antibody (MAb), immunoprecipitations were carried out with ³⁵S-labelled hAd5-cell extracts. Monoclonal antibodies were individually reacted with [³⁵S]-labelled hAd5-infected cell extracts. With the exception of F66-29F5-9-2 and F66-22B3-5-11 (cell culture-prepared MAb) all anti-hAd5 MAbs were derived from mouse ascites fluid. MAbs were categorized into 3 groups based on a comparison to an hAd5-infected cell extract reacted with anti-hAd5 polyserum (Fig. 6.2, Lane 1). MAb were specific for hexon protein (Lanes 2 to 6: F66-8B12-4-11, F66-15G6-7, F66-19G11-4-5, F66-21E7-4-5, F66-29F5-9-2), for fiber (Lanes 7 to 12: F66-1C5-5-2, F66-7G1-8-10, F66-8C6-7-3, F66-8C6-7-11, F66-22B3-5-6, F66-22B3-5-11), and for pVII, an 18 kD virion major core protein (Lane 13: F66-2C12-10-8). MAbs specific for fiber coimmunoprecipitated low amounts of penton base. In western blot analysis (data not shown) I detected MAb binding of hexon protein but failed to observe mAb binding to fiber or penton base.

Others have characterized MAbs against adenoviral type-, group-, intergroup-, and subgroup-specific determinants (reviewed in the INTRODUCTION). To determine if the monoclonal antibodies recognize determinants common to group C adenoviruses I reacted [³⁵S]-labelled hAd2-infected cell extracts with the MAb panel (Fig. 6.2, B). MAbs directed against hAd5 hexon as well as F66-2C12-10-8 (anti-protein VII) brought down the corresponding hAd2 proteins and were classified as group-specific, while MAbs reacted with hAd5 fiber were type-specific failing to precipitate hAd2 fiber protein.

ANTI-FIBER MONOCLONAL ANTIBODIES NEUTRALIZE AdlacZ INFECTIVITY

The recognition of antigenic determinants on hexon or fiber molecules can effectively neutralize adenovirus infectivity (reviewed in the INTRODUCTION). The infection of cells with AdlacZ can be monitored by measuring β -galactosidase expression. Adenoviral neutralization was measured by infecting cells with AdlacZ preincubated with antibody then assaying cell

FIGURE 6.2: IMMUNOPRECIPITATION OF hAd5 AND hAd2 STRUCTURAL PROTEINS

Immunoprecipitation of hAd5- and hAd2-infected HeLa cells with the monoclonal antibody (MAb) panel. A, hAd5-infected HeLa cells; B, hAd2-infected HeLa cells. Lane 1, rabbit anti-hAd5 polyclonal antiserum; Lane 2 through 13, MAb panel samples: 2, F66-8B12-4-11; 3, F66-15G6-7; 4, F66-19G11-4-5; 5, F66-21E7-4-5; 6, F66-29F5-9-2; 7, F66-1C5-5-2; 8, F66-7G1-8-10; 9, F66-8C6-7-3, 10, F66-8C6-7-11; 11, F66-22B3-5-6; 12, F66-22B3-5-11; 13, F66-2C12-10-8. Marker, [³⁵S]-labelled VSV Indiana proteins.

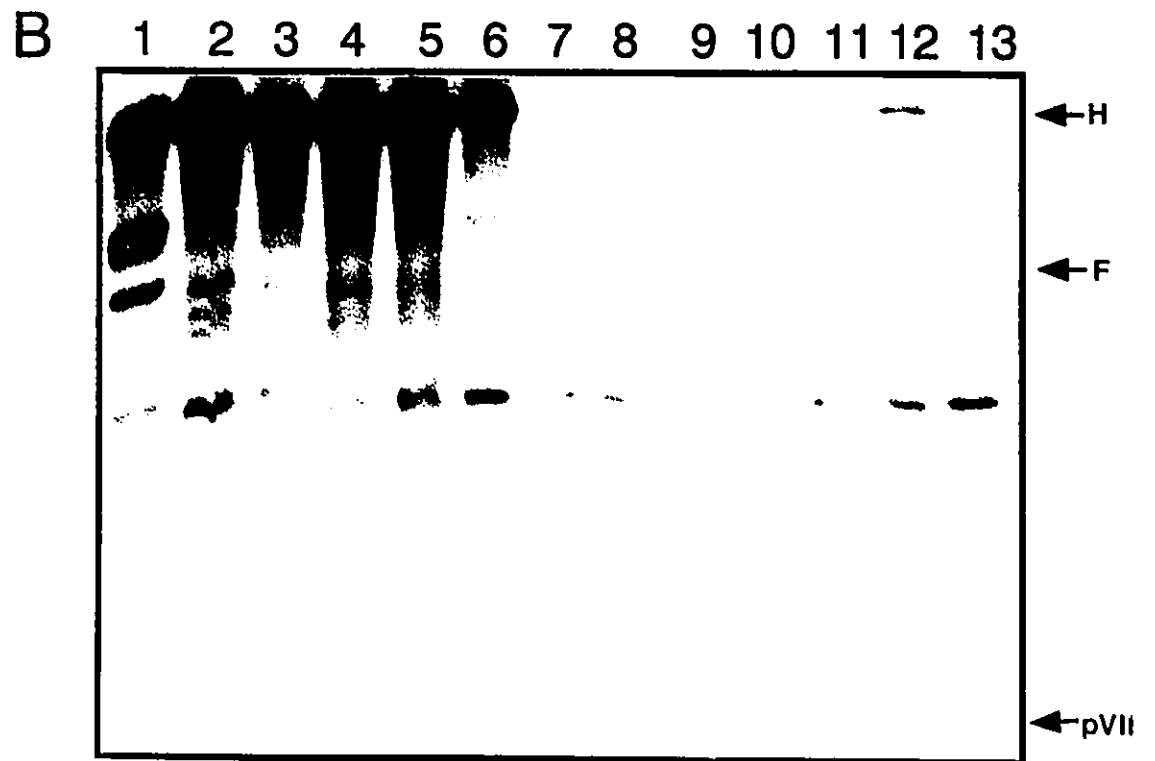
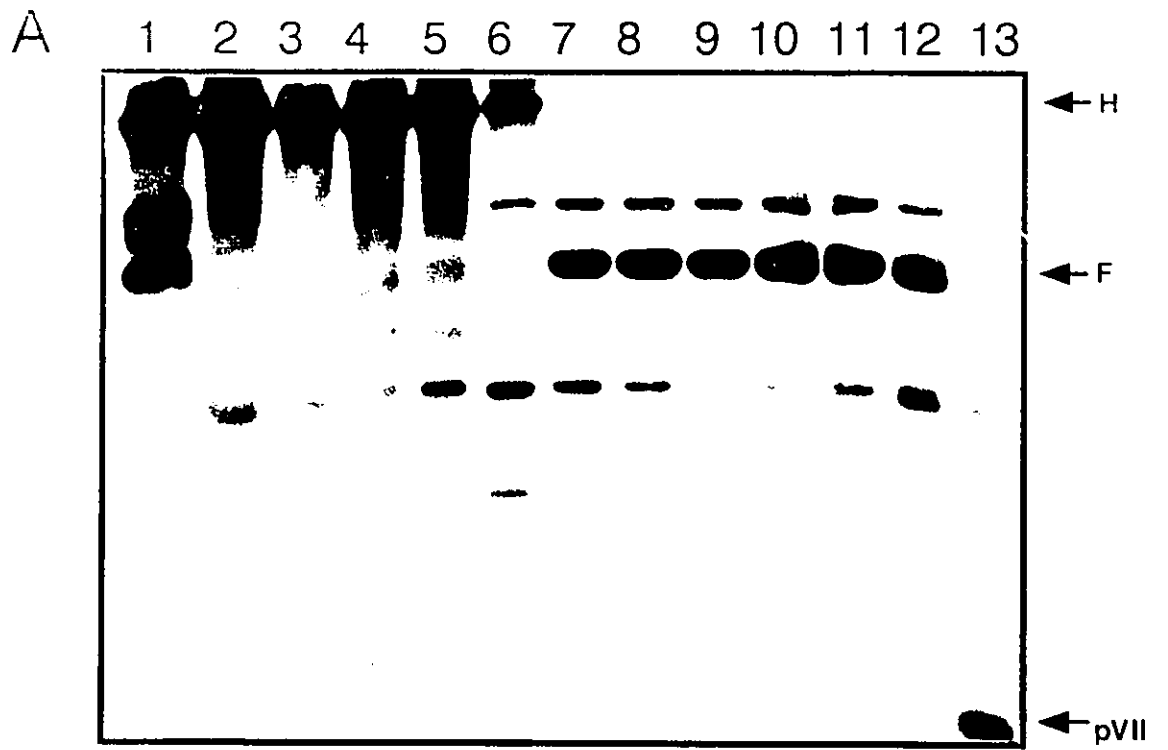


TABLE 6.1: CHARACTERIZATION OF MAbs DIRECTED AGAINST hAd5

Monoclonal Antibody	Identity	Neutralization of AdlacZ	Neutralizing dilution ¹
F66-8B12-4-11	hexon	negative	< 10
F66-15G6-7	hexon	negative	< 10
F66-19G11-4-5	hexon	negative	< 10
F66-21E7-4-5	hexon	negative	< 10
F66-29F5-9-2	hexon	negative	< 10
F66-1C5-5-2	fiber	positive	3750
F66-7G1-8-10	fiber	positive	250
F66-8C6-7-3	fiber	positive	> 6250
F66-8C6-7-11	fiber	positive	> 6250
F66-22B3-5-6	fiber	positive	> 6250
F66-22B3-5-11	fiber	positive	> 6250
F66-2C12-10-8	pVIII	negative	< 10

¹ Highest reciprocal antibody dilution resulting in a 50% decrease in β -galactosidase activity during AdlacZ neutralization.

extracts for β -galactosidase cleavage of the substrate *o*-nitrophenyl β -D galactopyranoside (ONPG). The colorimetric cleavage reaction was monitored at an optical density of 420 nm. MAbs type-specific for hAd5 fiber neutralized hAd5 infectivity while MAbs against hexon and pVII failed to prevent infection (Table 6.1). Anti-fiber clones F66-8C6-7-3, F66-8C6-7-11, F66-22B3-5-6, and F66-22B3-5-11 showed the highest neutralizing activity.

DISCUSSION

I have characterized the binding specificity of twelve monoclonal antibodies. Six MAbs were specific for the non-neutralizing group determinant on the hexon molecule. Five MAbs detected the type-specific determinant on fiber and neutralized adenoviral infection while one non-neutralizing MAb recognized core pVII.

MAbs F66-8B12-4-11, F66-15G6-7, F66-19G11-4-5, F66-21E7-4-5, and F66-29F5-9-2 detected group C hexon. Although the ability of the MAbs to detect hexon molecules of other

group was not determined, Wandeler has indicated that MAbs F66-19G11-4-5, F66-21E7-4-5, and F66-29F5-9-2 bind canine adenovirus (A. Wandeler, personal communication). Norrby showed that the hexon group determinant is not exposed on the virion surface (Norrby *et al.*, 1969a). Using fluorescence antibody labelling of infected cells Russell's group (1981) detected the hexon group determinant in the cytoplasm and not on the mature protein found in the nucleus. Wandeler has confirmed this labelling pattern for the anti-hexon MAbs described in this chapter (A. Wandeler, personal communication).

Hexon contains group-, subgroup-, or type-specific antigenic determinants (Norrby and Wadell, 1969; Russell *et al.*, 1981; Adám *et al.*, 1988) where type-specific determinants induce the production of neutralizing antibodies (Kjéllen and Pereira, 1968; Pereira and Laver, 1970; Waddell, 1972; Willcox and Mautner, 1976a, 1976b) while group- and subgroup-specific determinants do not stimulate production of neutralizing antibody (Haase and Pereira, 1972). The type determinant promotes low levels of virus aggregation by neutralizing antibodies (Wohlfart *et al.*, 1985; Wohlfart, 1988): significant levels of neutralization are obtained at reciprocal serum dilutions of 1.25 and 2 when added to 4.4×10^{10} virus. In my neutralizing assay the lowest reciprocal serum dilution used was 10 added to 6×10^5 pfu of AdlacZ which supports the non-neutralizing capabilities of the anti-hexon mAbs.

My results indicate that the monoclonal antibodies specific for fiber detect a neutralizing type-specific determinant. The type-specific determinants, found on the carboxy-terminus knob of the molecule (Norrby, 1969a), are responsible for cellular receptor binding (Pereira and de Figueiredo, 1962) and promote virus neutralizing activity (Wilcox and Ginsberg, 1963b; Valentine and Pereira, 1965; Norrby, 1966b, 1968; Kjéllen and Pereira, 1968; Wadell and Norrby, 1969; Haase and Pereira, 1972; Wöhlhart *et al.*, 1985; Watson *et al.*, 1988; Wöhlhart, 1988). MAbs specific for Ad5 fiber neutralized AdlacZ infection and failed to immunodetect Ad2 fiber supporting a specificity for the type determinant. In fluorescence antibody labelling experiments with these monoclonals, Wandeler localized mature fiber to the nucleus (A. Wandeler, personal communication). Fiber and penton base are found in a noncovalent state at the virion vertices. The ability of anti-fiber MAbs to coimmunoprecipitate penton base may reflect the procedure used to disrupt the viral components and has been similarly reported by Russell *et al.* (1981). The variability in reciprocal serum dilutions required to neutralize AdlacZ

suggests that at least two different type-specific determinants are recognized. The exact breakdown of the type-specific determinants can be assessed in competition studies using ¹²⁵I-labelled mAb preparations.

pVII, also known as core protein 1, is found associated with viral DNA in a nucleosome-like structure (Russell *et al.*, 1971; Everitt *et al.*, 1973, 1975) and carries both group- and type-specific antigenic determinants (Prage and Pettersson, 1971). Immunodetection of pVII with MAb F66-2C12-10-8 in Ad2-infected cells confirms the specificity of clone F66-2C12-10-8 for the common group determinant.

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