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IN VITRO AND *IN VIVO* CHARACTERIZATION OF THE HEXON OF HEMORRHAGIC ENTERITIS VIRUS OF TURKEYS (TYPE II AVIADENOVIRUS)

By

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ABSTRACT

IN VITRO AND *IN VIVO* CHARACTERIZATION OF THE HEXON OF HEMORRHAGIC ENTERITIS VIRUS OF TURKEYS (TYPE II AVIADENOVIRUS)

By

Carol J. Cardona

The structure of the icosahedral adenovirus capsid is highly conserved among Adenoviridae. In its native form, the hexon is the major capsid protein. The nascent hexon requires the 100 kD folding protein to fold into its native, trimeric form but may also require other adenoviral proteins. The hexon and 100 kD folding protein genes were identified in the HEV genome, cloned, and sequenced. The hexon and 100 kD folding proteins were then cloned into and co-expressed in a fowlpox virus (FPV) vector. In the recombinant FPVs (rFPVs) in which the hexon and 100 kD folding protein genes were cloned head to tail, the native hexon could be detected. Expression of the nascent hexon and the 100 kD folding protein

were confirmed in all rFPVs with Western blotting and detection with polyclonal turkey anti-HEV serum. The rFPVs expressing both the hexon and 100 kD folding protein were tested in chickens for their ability to elicit a humoral immune response. The FPV-@X100 construct in which the 100 kD folding protein gene follows the hexon gene head to tail, elicited the largest response. The anti-HEV humoral immune response in turkeys inoculated with FPV-@X100 was compared to the humoral response of turkeys given a commercial HEV vaccine. The humoral immune responses elicited by the two vaccines were indistinguishable at most times. However, after 35 days, the rFPV anti-HEV titers were significantly lower than the antibody titers elicited by the commercial vaccine.

The rFPV expressing the native hexon of HEV was compared to the commercial HEV vaccine for its ability to protect turkeys from virulent HEV challenge. Complete protection from the intestinal lesions of HE was achieved in experimental groups vaccinated with either the rFPV or the commercial vaccine. Lymphocyte stimulation was measured in turkeys inoculated with rFPV and stimulation indices were not significantly different from the results observed in uninoculated turkeys.

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KEY TO ABBREVIATIONS

2YT	two yeast-tryptone
AASV	avian adenosplenomegaly virus
Ad	human adenovirus
BA	*Bordetella avium*
BAV	bovine adenovirus
BCIP	5-bromo-4-chloro-3-indolylphosphate p-toluidine salt
bp	base pairs
C	Centigrade
CAV	canine adenovirus
CEF	chick embryo fibroblast
CELO	chick embryo lethal orphan
CIAV	chicken infectious anemia virus
ConA	concanavalin A

cpm	counts per minute
D	dalton
DBP	DNA binding protein
DEF-A	downstream element factor A
DEF-B	downstream element factor B
DMSO	dimethyl sulfoxide
DNA	dioxyribonucleic acid
ds	double stranded
E. coli	*Escherichia coli*
EAV	equine adenovirus
EDSV	egg drop syndrome 76 virus
EDTA	ethylenediaminetetraacetic acid
EEV	extracellular enveloped virion
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbant assay

FAV	fowl adenovirus
FITC	flourescein isothiocyanate
FPV	fowlpox virus
GALT	gut associated lymphoid tissue
HE	hemorrhagic enteritis
HEV	hemorrhagic enteritis virus
IBDV	infectious bursal disease virus
IBH	inclusion body hepatits
IgG	immunoglobulin G
IgM	immunoglobulin M
INV	intracellular naked virion
IPTG	isopropyl β-D-thiogalactopyranoside
ITR	internal terminal repeat
kb	kilobases
kD	kilodalton

LM	Leibovitz-McCoy medium
LVD	leucine-valine-aspartic acid
MAb	monoclonal antibody
MAV	murine adenovirus
MDV	Marek's disease virus
MHC	major histocompatibility complex
min.	minutes
μCi	micro Curie
ml	milliliter
μl	microliter
MLP	major late promoter
MLTU	major late transcription unit
μm	micrometer
mM	millimolar
moi	multiplicity of infection

mRNA	message RNA
MSDV	marble spleen disease virus
NBT	nitroblue tetrazolium chloride
NDV	Newcastle disease virus
ng	nanogram
nm	nanometers
nts	nucleotides
OAV	ovine adenovirus
ORF	open reading frame
PAV	porcine adenovirus
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units
PHA	phytohemagglutinin
pi	post inoculation

picoM	picomole
pTP	pre-terminal protein
QBV	quail bronchitis virus
r-strand	rightward transcribed strand
rFPV	recombinant fowlpox virus
RGD	arginine-glycine-aspartic acid
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
sec.	seconds
SPF	specific pathogen free
SPF	specific pathogen free
ss	single stranded
SSC	sodium trisodium citrate
TBS	Tris buffered saline

TCID	tissue culture infective dose
TE	Tris-EDTA
TK	thymidine kinase
TNF-α	tumor necrosis factor alpha
TP	terminal protein
ts	temperature sensitive
U	international unit
VA	virus associated
vHEV	virulent hemorrhagic enteritis virus
vxHEV	vaccine strain hemorrhagic enteritis
X	times

Chapter 1

LITERATURE REVIEW

I. Adenoviruses

The Adenoviridae are a large and diverse family of viruses which are divided into two genera: mastadenoviruses and aviadenoviruses (Wigand et al., 1982). The mastadenoviruses have a mammalian host range while the aviadenoviruses infect avian species. The separation of these genera is based on the presence or absence of common group-specific, complement fixing antigens (Monreal, 1992).

Adenoviruses are non-enveloped viruses with icosahedral symmetry, 70-90 nm in diameter capsid, and a linear double stranded DNA (dsDNA) viral genome (Wigand et al., 1982). Adenoviruses weigh between 170 and 175 x 10^6 daltons (D) molecular weight and have at least ten polypeptides which range in size from 5 x 10^3 kilodaltons (kD) to 120 x 10^3 kD (Grodzicker et al., 1977, Wigand et al., 1982). The molecular weight of chick embryo lethal orphan (CELO) virus is estimated to be 173 x 10^6 D

which falls into the range estimated for human adenoviruses. The molecular weight of CELO virus DNA is 30 x 10^6 D (Laver et al., 1971). The molecular weights of human adenovirus genomes are 20 to 25 x 10^6 D. The DNA of egg drop syndrome 76 virus (EDSV) weighs 22.9 x 10^6 D (Monreal, 1992).

The type I aviadenoviruses, including the prototype aviadenovirus, CELO virus, have larger genomes than mastadenoviruses (Sussenbach, 1984). CELO virus has a genome of 43.8 kilobases (kb) (Chiocca et al., 1996), slightly larger than mastadenoviral genomes which range from approximately 30 kb to 36 kb. Another type I aviadenovirus, fowl adenovirus 8 (FAV 8), has a genome size estimated to be 44.7 kb (Clavijo et al., 1996). In contrast to the type I aviadenoviruses, the type II and type III aviadenovirus genomes fall within the mastadenovirus size range. The genomes of type II aviadenoviruses, including hemorrhagic enteritis virus (HEV) of turkeys, are approximately 25 kb in length (McQuiston et al., 1995, McFerran et al., 1997, Jucker et al., 1996). The type III aviadenovirus, EDSV, has a genome of 33.4 kb (Brandt et al., 1997).

A. Avian adenoviruses

Mastadenoviruses are defined as distinct species based on having 1) unrelated hemagglutinins or 2) substantial biophysical or biochemical differences (Wigand et al., 1982). The avian adenoviruses are not defined as distinct species based on hemagglutinin characteristics since most are non-hemagglutinating viruses. Most aviadenoviruses have traditionally been defined as distinct species based on pathogenicity for a specific target host. This approach is somewhat limited since many isolates have overlapping host ranges (Monreal, 1992).

The aviadenoviruses are subdivided into three groups on the basis of group-specific antigen reactions. Group I or type I aviadenoviruses share a common group antigen. Group II or type II aviadenoviruses share a group antigen distinct from the group antigen of type I aviadenoviruses. Group III or type III aviadenoviruses partially share the type I group antigen (McFerran et al., 1997).

Aviadenoviruses have been reported in a variety of tissue types in several avian species. Adenoviral infections are well known in chickens, turkeys, quail, pheasants, ducks, geese, and guinea fowl. Other avian species in which adenoviral infections have been reported include pigeons

(Goryo et al., 1988), a variety of psittacines (Mori et al., 1989, Ramis et al., 1992, Capua et al., 1995), kestrels, ostriches, herring gulls, the common murre, and a tawny frogmouth (McFerran et al., 1997).

1. Type I aviadenoviruses

The type I aviadenoviruses (fowl adenoviruses {FAVs}) have broad antigenicity (Cowen et al., 1977) which has led to some disagreement about the serotype classification of some isolates. Twelve fowl serotypes have been recognized and there may be others which have not yet been classified (Calnek and Cowen, 1975, McFerran and Connor, 1977, McFerran et al., 1997). FAV serotypes have been divided into five groups using DNA restriction pattern analysis (Monreal, 1992).

The type I aviadenoviruses have been associated with a variety of disease syndromes. In recent decades, the role of type I aviadenoviruses as primary pathogens has been open to question. Adding to this quandry is the isolation of type I aviadenoviruses from healthy chickens (Yates et al., 1976). It now appears that some of the lesions attributed to type I aviadenoviruses might have been caused by agents such as chicken infectious anemia virus (CIAV) (Yuasa et al., 1979) and infectious bursal

disease virus (IBDV) (Dhillon, 1986). For example, the aplastic anemia associated with inclusion body hepatitis (IBH) and the bursal lesions of the same syndrome were probably caused by CIAV and IBDV, respectively. With respect to this dilemma, the following is a summary of disease syndromes associated with type I aviadenoviruses.

Respiratory disease in chickens. Mild to moderate catarrhal tracheitis has been attributed to FAV infection in natural outbreaks. Histologically, the major lesions observed were tracheal deciliation, necrosis of tracheal epithelial cells, and infiltration of mononuclear inflammatory cells into the lamina propria of the trachea (McFerran et al., 1997).

Inclusion body hepatitis in chickens. The major lesions of IBH are confined to the liver which is pale, friable, and swollen (Winterfield et al., 1973). Intranuclear inclusions are readily observable in hepatocytes (Gallina et al., 1973). Hydropericardium may also be observed in cases of IBH. Outbreaks of IBH independent of IBDV involvement have been reported in New Zealand (Christensen and Saifuddin, 1989) and Australia (Erny et al., 1991).

Pancreatitis and gizzard erosions. Focal pancreatitis and gizzard erosions have been associated with type I aviadenoviruses in chickens (Tanimura et al., 1993) and guinea fowl. Intranuclear inclusion bodies have been observed in pancreatic acinar cells (Tanimura et al., 1993, McFerran et al., 1997).

Quail bronchitis. Quail bronchitis causes an acute respiratory disease in quail less than 3 weeks of age. Mortality in affected flocks may reach 60%. The respiratory system is the most severely affected with the trachea and bronchi being the target organs. Grossly, the tracheal mucosa may be thickened and covered with moist, necrotic, and sometimes hemorrhagic exudate (Jack and Reed, 1990). Splenomegaly or splenic mottling have been observed in quail experimentally inoculated with quail bronchitis virus (QBV) at 6-9 weeks of age. Histologically, the tracheal lesions may range from deciliation and proliferation to necrosis and desquamation. Intranuclear inclusions can be observed in tracheal epithelial cells. Bronchi may be similarly affected but with greater inflammatory cell infiltration (Jack and Reed, 1990). Histologically the splenic lesion is described as hyperplasia of splenic macrophages (Jack and Reed, 1990). Multifocal hepatocellular necrosis with large basophilic intranuclear

inclusions may also be observed (Jack and Reed, 1987, McFerran et al., 1997; Jack and Reed, 1990). Gross atrophy of the bursa of Fabricius and histologic lesions including individual cell necrosis and intranuclear inclusions in the bursal epithelium have been described (Jack and Reed, 1990). Interestingly, QBV is serologically indistinguishable from CELO virus (FAV-1) (Dubose and Grumbles, 1959, Yates and Fry, 1957), and other type I aviadenovirus isolates (Jack and Reed, 1987). An adeno-associated virus-like virus was reported associated with QBV in a single report (Dutta and Pomeroy, 1967).

Isolates of FAVs have been made from the respiratory, gastrointestinal, and urinary systems from turkeys with acute respiratory disease. Inoculation of most of these viruses into susceptible turkeys has confirmed that they are either non-pathogenic or require other predisposing factors to cause disease (Sutjipto et al., 1977). A case of inclusion body hepatitis in turkeys caused by a suspected type I aviadenovirus has been reported (Guy et al., 1988). Several different serotypes of FAVs have been isolated from turkeys but a classification of serotypes has not yet been fully determined (Easton and Simmons, 1977, McFerran et al., 1997).

CELO virus is oncogenic and can both transform cells in culture

(Ishibashi et al., 1987) and produce fibrosarcomas or sarcomas at the site of injection in newborn hamsters. Hepatomas, adenocarcinomas, and sarcomas in the livers, and ependymomas in the brains of newborn hamsters have also been reported (Sarma et al., 1965, Stenback et al., 1973, Fadly et al., 1976, Dhillon and Jack, 1997). Most authors agree that only the Phelps CELO strain (FAV 1) can induce tumors in hamsters despite the high level of cross reactivity between type I aviadenoviruses (Fadly et al., 1976). However, a recent report indicates that other FAV 1 isolates may also be oncogenic in non-target species (Dhillon and Jack, 1997). One type I aviadenovirus isolate, DPI-2, has been reported to cause hepatitis similar in appearance to inclusion body hepatitis of chickens when inoculated into hamsters (Fadly et al., 1976).

2. Type II aviadenoviruses

There are three type II aviadenoviruses: marble spleen disease virus (MSDV) of pheasants, avian adenovirus splenomegaly virus (AASV) of chickens, and hemorrhagic enteritis virus (HEV) of turkeys (Domermuth and Gross, 1991, McFerran et al., 1997). Serologically, there is high cross reactivity between these viruses. MSDV, AASV, and HEV can cause rapid

death in their respective target species, but are of low pathogenicity in non-target species (Fadly et al., 1988, Domermuth and Gross, 1991, McFerran et al., 1997). Several species of psittacine birds (Gomez-Villamandos et al., 1995) and guinea fowl (Cowen et al., 1988, Massi et al., 1995) have been reported with hemorrhagic enteritis caused by suspected type II avian adenoviruses. These suspected type II aviadenoviruses have neither been isolated nor characterized. The type II aviadenoviruses can be differentiated from one another on the basis of host range, with restriction endonuclease fingerprinting (Zhang and Nagaraja., 1989), and with monoclonal antibodies (van den Hurk and van Drunen Littel-van den Hurk, 1988, Zhang et al., 1991, van den Hurk, 1992).

Marble spleen disease was first recognized in Italy in 1966 (Mandelli et al., 1966). It is a disease of intensively raised pheasants usually 4-8 months of age. The clinical signs of MSD are usually absent due to the peracute onset of the disease. However, when clinical signs are observed, they consist of slight depression, dyspnea, and finally, asphyxia. Mortality in affected flocks may be 5-15% (Domermuth et al., 1979a). Grossly, spleens are 2-3 times normal size with a mottled appearance. Notable is severe pulmonary edema, which is the fatal lesion. Histologically, the

splenic lesion is characterized by lymphoid depletion, fixed-tissue macrophage hyperplasia, and intranuclear inclusions in mononulcear phagocytic cells (Fitzgerald and Reed, 1991).

Avian adenovirus splenomegaly virus was first isolated from broilers in the United States in the mid 1970s (Domermuth et al., 1979b). This virus was found to be antigenically indistinguishable from HEV and MSDV (Domermuth et al., 1980). Based on serologic surveys, infection appears to be widespread in both broiler and layer populations in North America (Domermuth et al., 1980). Morbidity in infected flocks averages 1-4% and mortality is usually insignificant. There is one report of an outbreak of AASV in 20 week old broilers in which there was 8.9% mortality over the 10 days of the outbreak (Domermuth et al., 1982). Similar to MSDV, deaths from AASV are due to severe pulmonary edema. In fatal cases, gross lesions of splenomegaly, severe pulmonary congestion and edema, hepatomegaly and hydropericardium have been reported (Domermuth et al., 1982). Histologically, viral intranuclear inclusions can be found in mononuclear phagocytic cells, usually in the spleen (Domermuth et al., 1979b, Veit et al., 1981). Interestingly, both experimental and natural infections of AASV can only be detected after concentration of the virus via

serial passages through susceptible turkeys indicating that the virus exists in the chicken in very low concentration (Domermuth et al., 1979b, Domermuth et al., 1982, Veit et al., 1981).

3. Type III aviadenoviruses

One serotype of EDSV and three genotypes of EDSV are recognized. The genotypes are divided as follows: 1) isolates from chickens in Europe, 2) isolates from ducks in the United Kingdom, and 3) isolates from Australian chickens (McFerran et al., 1997).

Ducks are likely to be the natural host of EDSV (Monreal, 1992). EDSV has been isolated from normal ducks and many duck flocks have EDSV antibodies. Infection with EDSV is also common in geese. EDSV was probably introduced into the commercial chicken population through a contaminated vaccine (McFerran et al., 1997).

Egg drop syndrome (EDS) is primarily a disease of broiler breeder or layer chickens and experimentally EDSV has no predilection for breed or strain. The first sign of infection with EDSV is a loss of color in pigmented eggs, followed by the laying of thin-shelled or shell-less eggs. Outbreaks usually last 4-10 weeks and egg production can be reduced by up to 40%

during an outbreak. Usually, however, any lost production is made up by an increased rate of lay late in the lay cycle so that losses are minimized. Grossly, inactive ovaries and atrophied oviducts are reported. Histologically, intranuclear inclusions are consistently observed in the epithelial cells of the pouch shell gland 7 days after infection. Intranuclear inclusions are seen in epithelial cells of the infundibulum, tubular shell gland, pouch shell gland, isthmus, sinus, and in the spleen of experimentally infected chickens. The lamina propria of the shell gland may have a moderate to severe mononuclear inflammatory reaction (McFerran et al., 1997).

II. Hemorrhagic enteritis of turkeys

A. History.

Hemorrhagic enteritis of turkeys was first described in 1937 by Pomeroy and Fenstermacher (Pomeroy and Fenstermacher, 1937). These first outbreaks occurred in 35 turkeys, 7-12 weeks old from widely separated and variously sized flocks in Minnesota. Severe hemorrhagic enteritis most severe in the duodenum was described as well as widely scattered hemorrhages in many organ systems and an overall anemic

appearance. Gale and Wyne reported the next two outbreaks of HE in 1957 in Ohio although they reported that HE had occurred sporadically in the intervening 20 years (Gale and Wyne, 1957). HE emerged and reached epidemic proportions in Texas in the early 1960s and in Virginia in the mid-1960s (Gross and Moore, 1967, McFerran et al., 1997).

Gross described the lesions of HE in 1967. In that work, the timing of gross and histologic lesions of the intestine were described (Gross, 1967). The disease was determined to be transmissible with filtered and unfiltered intestinal contents and sera from infected turkeys (Gross, 1967, Domermuth and Gross, 1972). However, it was not until 1974 that the characteristic intranuclear inclusions were observed and an adenovirus isolated by Carlson et al. Electron microscopy showed the virions in three forms in intranuclear inclusions: loose virus particles, extranuclear fibrous inclusions, and large arrays of virus crystals. The virus particles were icosahedral and 70-75 nm in diameter. The virus was tentatively classified as an adenovirus at this time (Carlson et al., 1974). The virus was later classified as a type II aviadenovirus (Domermuth et al., 1980).

B. Lesions of hemorrhagic enteritis in turkeys

Though this disease is named for the prominent enteric lesions it induces, splenic lesions are a more consistent feature of HE. The spleen is the primary site of viral replication and, as such, contains the greatest amount of virus (Gross and Domermuth, 1976, Carlson et al., 1974, Itakura and Carlson, 1975a, Itakura and Carlson, 1975b, Tolin and Domermuth, 1974, Silim et al., 1979, Ossa et al., 1983b). Characteristically, the spleen is enlarged, three to four times normal size, and mottled (Domermuth and Gross, 1991, Gross and Domermuth, 1976, Itakura and Carlson, 1975b). The mottled appearance is due to two factors: 1) congestion of splenic red pulp and 2) white pulp hyperplasia (Gross and Domermuth, 1976). In experimentally inoculated poults, spleen size increased until day 4 post inoculation (pi) after which it gradually resumes its normal dimension by day 24 pi (Gross and Domermuth, 1976). The spleens of dead poults are smaller and less marbled due to blood loss and subsequent splenic contraction (McFerran et al., 1997). Based on these feature, splenomegaly is a more reliable indication of HEV infection than are intestinal lesions (Gross and Domermuth, 1976, Itakura and Carlson, 1975a, Itakura et al., 1974, Ossa et al., 1983a, Itakura and Carlson, 1975b).

Histologically, the splenic lesions are characterized by lymphoid necrosis and red pulp congestion which can be observed as early as 6 hours pi. Twenty-four hours pi, intranuclear inclusions typical of HEV infection first appeared in splenic macrophages in the white pulp. Intranuclear inclusions are large, homogenous, elliptical, 5.6-11.6 μm in diameter, and fill the nucleus (Fujiwana et al., 1974). At 3 days pi, the white pulp is hyperplastic with increased numbers of mitotic figures (Itakura and Carlson, 1975b, Gross and Domermuth, 1976, Domermuth and Gross, 1991, Saunders, 1993). Degeneration and necrosis of lymphoid cells and reticular cells of the white pulp is a feature of HE (Gross and Domermuth, 1976). There is a positive correlation between lymphoreticular hyperplasia, the appearance of inclusions, and peak virus precipitating antigen production in the spleen (Gross and Domermuth, 1976). By days 6-8 pi, the splenic architecture has returned to normal (Gross and Domermuth, 1976).

The clinical signs of classical, naturally occurring HE are depression, bloody droppings, and rapid death (Gross, 1967, Itakura and Carlson, 1975b, Silim and Thorsen, 1981, Domermuth and Gross, 1991). These signs are primarily due to the massive intestinal bleeding associated with classical HE. Duration of blood loss from the gut occurs over a 24 hour

period. Birds that died a day after passing bloody droppings had no blood in their intestines at necropsy (Gross and Moore, 1967). The signs of HE may include other non-specific signs of enteritis, i.e., flushing, wet litter, and high pitched crying (Gross and Domermuth, 1976, Domermuth and Gross, 1991). There is often feed in the crop and gizzard of dead poults indicating the course of the disease is short.

Intestinal lesions appear on the day after viral antigen concentration peaks in the spleen (Gross and Domermuth, 1976, Silim and Thorsen, 1981, Ossa et al., 1983a). The intestinal lesions most characteristic of HE are confined to the small intestine, particularly the duodenum just distal to the entrance of the pancreatic ducts (Gross, 1967, Itakura and Carlson, 1975b, Itakura et al., 1974, Silim and Thorsen, 1981, Domermuth and Gross, 1991, Saunders et al., 1993). The earliest histologic change is congestion of the capillaries of the villus tips in the duodenum and jejunum 5 days after oral inoculation with infective virus (Gross and Moore, 1967, Gross, 1967, Saunders et al., 1993). Congestion increases and there is rapid diapedesis of erythrocytes and leakage of protein rich fluid from the vessels of the lamina propria (Gross, 1967). Macrophages, plasma cells, and heterophils infiltrate the lamina propria and intranuclear inclusions are evident in

macrophages (Saunders et al., 1993). Varying degrees of lymphocytic hyperplasia are associated with the presence of large mononuclear cells containing intranuclear inclusions (Itakura and Carlson, 1975a).

Late on the 5th day, the mucosal epithelium lifts away from the underlying lamina propria (Gross, 1967, Silim and Thorsen, 1981). This separation allows blood from the lamina propria to flow into the intestinal lumen. In severely affected birds, the tips of the intestinal villi become necrotic and slough into the intestinal lumen on day 6 pi (Gross, 1967, Silim and Thorsen, 1981). Grossly the duodenum and jejunum, are distended with blood and necrotic intestinal mucosa (Gross, 1967, Itakura and Carlson, 1975b, Silim and Thorsen, 1981, Domermuth and Gross, 1991). Heterophils have been described at the juncture of necrotic and viable tissue in acute HE infection (Gross, 1967, Domermuth and Gross, 1991, Opengart et al., 1992, Saunders et al., 1993). This influx of heterophils is probably secondary to active necrosis and not a direct effect of viral infection (Cotran et al., 1989).

By the middle of the 6th day, the mucosal epithelium reforms and hemorrhage into the intestinal lumen ceases (Gross, 1967). Macrophages with hemosiderin appear in the lamina propria on day 7 pi. The capillaries

of the lamina propria remain congested until day 7-9 pi (Gross, 1967). Ten days after inoculation, nearly all signs of infection had disappeared except for a small amount of fibrosis at the tips of villi which were sloughed (Gross, 1967).

Lesions similar to those described in the duodenum and jejunum, may occur in the proventriculus, ventriculus, ileum, large intestine, and cecae (Itakura and Carlson, 1975b, Saunders et al., 1993). Lesions similar to the splenic lesions may also occur in the bursa of Fabricius, and cecal tonsils (Itakura and Carlson, 1975b, Saunders et al., 1993). Hepatic necrosis has been described in turkeys with HE (Wilcock and Thacker, 1976). Intranuclear inclusions associated with HEV infection have been described in renal tubular cells without apparent necrosis or inflammation (Silim and Thorsen, 1981, Meteyer et al., 1992, Trampel et al., 1992).

There is both the overtly pathogenic form of HE and a considerably milder form characterized by only splenomegaly and seroconversion. Mortality in field outbreaks of HEV infection range from 60% for the overtly pathogenic form to 0.1% for the milder form over the course of the disease outbreak (McFerran et al., 1997). Both manifestations of HEV infection cause economic loss. Both forms of the disease can cause

diminished rates of gain and reduced feed conversion which decrease profits in raising commercial turkeys. But, more importantly HEV causes immunosuppression which prevents turkeys previously infected with HEV from mounting an effective immune response against opportunistic infections (Nagaraja et al., 1982a, Nagaraja et al., 1982b and Nagaraja et al., 1985, Newberry et al., 1993, Larsen et al., 1985, Sponenberg et al., 1985, van den Hurk et al., 1994). The most important of these opportunistic organisms is *Escherichia coli* (*E. coli*). Colibacilosis (or *E. coli* infection) causes losses directly in deaths and reduced weight gain as well as in increased condemnations at the time of slaughter. HEV in combination with other pathogens including *Bordetella avium* (BA), Newcastle disease virus (NDV), and *Mycoplasma meleagridis* has been shown to predispose turkeys to *E. coli* infection in the field (Pierson et al., 1996). Experimentally, a synergistic effect on mortality and the incidence of pericarditis was demonstrated by infection of 4 week old poults with NDV, BA, HEV, and *E. coli*. The timing of the administration of these multiple agents may influence the magnitude of this effect (Pierson et al., 1996). Colibacilosis is not the only disease agent to which turkeys are more susceptible to after infection with HEV. Other reports indicate

susceptibility to 1) pneumovirus infection, and 2) chlamydiosis (Andral et. al., 1985). In addition, diminished responses to vaccines have been reported after HEV infection (Nagaraja et. al., 1985).

C. Pathogenesis of hemorrhagic enteritis.

Hemorrhagic enteritis virus can remain infectious in contaminated litter for several weeks or months and is most frequently transmitted by a fecal-oral route (McFerran et al., 1997). When it enters the gastrointestinal system, the HEV virion gains access to the gastrointestinal associated lymphoid tissue (GALT). Initially HEV replicates in the GALT, especially in the cecal tonsils. Experimentally, this has been demonstrated by the early appearance of HEV antigen in the cecal tonsils (Fasina and Fabricant, 1982, Suresh and Sharma, 1996). The cecal tonsils in turkeys are paired and lie at or near the ileo-cecal junction. The domed intestinal surface overlying the cecal tonsils is composed of a specialized mucosal epithelium, the lymphoepithelium (Lillehoj, 1996, Pope, 1996). The lymphoepithelium lacks a basement membrane and lymphocytes lie both between epithelial cells and in invaginations along the basal surface (Pope, 1996). Germinal centers with both B- and T- lymphocytes lie in the lamina propria of the

cecal tonsils. Most of the lymphocytes in the cecal tonsils are IgM+ lymphocytes (Lillehoj, 1996).

After replicating in the B-lymphocytes of the cecal tonsils, HEV then infects peripheral blood lymphocytes and can be detected in peripheral blood lymphocytes 4-8 days pi (Fasina and Fabricant, 1982). The virus localizes in the spleen where it begins extensive replication days 5-7 pi. HEV travels to the spleen via the splenic artery and trabecular arteries in the spleen. The splenic trabecular arteries give rise to smaller central arteries which branch into smaller penicilliform capillaries and finally open into the splenic red pulp. The red pulp is drained by collecting veins which join larger trabecular veins. The trabecular veins connect to the splenic vein which in turn connects to the vena cava. The vascular tree of the spleen is surrounded by the white pulp. The central arteries and draining veins are surrounded by periarteriolar sheaths of white blood cells primarily T-lymphocytes. The penicilliform capillaries of the vascular tree are surrounded by the macrophages and dendritic reticular cells which process antigen. The penicilliform capillaries are lined by endothelium characterized by intercellular channels which allow the outflow of blood borne antigens (Pope, 1996). This may be the point of entry for HEV into

the spleen. The periellipsoidal white pulp primarily composed of B-lymphocytes may be the initial splenic target for HEV replication. Suresh and Sharma (1996) were only able to detect HEV antigen in IgM+ B-lymphocytes in the spleen. The periellipsoid sheath is surrounded by macrophages which may also become infected with HEV. The periellipsoidal macrophages may proliferate along with the splenic reticular cells or ellipsoid associated cells in the ellipsoid sheath. The hyperplasia of these white pulp elements is likely in response to the necrosis of B-lymphocytes as the virus lyses infected cells.

The underlying pathogenesis for the intestinal lesions of HE may be mast cell mediated. There are more mucosal mast cells in the duodenums of turkeys with HE lesions than in normal turkeys (Opengart et al., 1992). In addition, carbon labeling of vessels indicates that there is loss of vessel wall integrity in the duodenums of birds with HE lesions. The vasoactive mediator products of mast cells (histamine and serotonin) act on endothelial cells, leading to the loss of vessel wall integrity, loss of serum albumin, and erythrocytes (Opengart et al., 1992). In addition to the accumulation of mast cells in the intestines of turkeys with HE lesions, there is an overall decrease in serum albumin concentration in HEV infected turkeys (Soback

et al., 1985). This is another potential mechanism for the formation of edema fluid, however, hypoproteinemia, while undoubtedly a significant factor in the formation of lesions in HE, would produce a generalized edema rather than just enteric edema (Cotran et al., 1989).

Thalidomide, a specific tumor necrosis factor-alpha (TNF-α) antagonist, administered to turkeys infected with HEV inhibited the development of HEV induced intestinal hemorrhages (Suresh, 1995). Turkey interferon administered to HE infected turkeys exacerbated the severity of HE intestinal lesions (Sharma and Rautenschlein, 1996). Treatment of turkeys with cyclosporin A prior to challenge with virulent HEV protected turkeys against HE intestinal lesions suggesting a pivotal role for T-lymphocytes (Suresh, 1995). Cyclosporin A treatment specifically causes the depletion of T-lymphocytes. In summary, the role of cytokines from macrophages and T-lymphocytes is not completely clear. However, it is clear that the intestinal lesions of HE are immune mediated and directly controlled by cytokines from activated T-lymphocytes and/or macrophages.

1. Host factors

There is a definite age associated resistance in turkeys to the development of HE. Three day old poults can be infected with HEV and the virus will replicate, however, the lesions of HE will not develop (Fadly and Nazerian, 1982). Turkeys vaccinated at 24 days of embryonation and at 1 day of age with MSDV had detectable viral antigen in spleen, liver, and intestine at 6 and 10 days of age (Ahmad and Sharma, 1993). However, poults experimentally infected with HEV when less than 3 weeks of age will not develop disease (Fadly and Nazerian, 1982). The youngest poults involved in a natural outbreak were 2.5 weeks old at the onset of clinical signs (Harris and Domermuth, 1977). HE has been produced in susceptible turkeys up to 52 weeks of age (Domermuth and Gross, 1991).

The pathogenesis of age resistance is partially but not fully explained by the presence of maternal antibody. Early resistance lasts longer in poults with maternal antibodies, however, poults without maternal antibodies are also resistant to developing the lesions of HE (Domermuth and Gross, 1991, Fadly and Nazerian, 1989). In turkeys infected with HEV at 6 weeks of age, the development of lesions was directly correlated to maternal antibody titers as measured at 2 weeks of age, though at 6 weeks of age maternal

antibody was undetectable (Fadly and Nazerian, 1989). Typically commercially raised turkeys have evidence of HEV infection at 6 to 8 weeks of age and seroconvert at 7 to 10 weeks of age in the field (Meteyer et al., 1992, McFerran et al., 1997).

Another clue in the quandary of age associated resistance to HE is the failure to produce lesions in bursectomized poults (Beasley and Wisdom, 1978, , Fadly and Nazerian, 1982). Bursectomized poults infected with virulent HEV failed to develop the gross or histologic lesions of HE in contrast to infected non-bursectomized poults which developed classical HE. Interestingly, HEV antigen was detectable in the spleens of HEV infected and bursectomized poults (Fadly and Nazerian, 1982). This work indicates the bursa of Fabricius is necessary for the pathogenesis of HE lesions but not for replication of the virus. Splenectomy has also been reported to prevent the lesions of HE in turkeys (Ossa et al., 1983a).

HEV infects all strains and breeds of commercial turkeys (Domermuth and Gross, 1991). One report suggests that four different genetic strains of turkeys differed in their responses to inoculation with virulent and attenuated HEV. The differences reported include the timing and severity of clinical signs and the timing of the onset of humoral

immunity (Le Gros et al., 1989). However, the turkeys used for this study were outbred strains of commercial turkeys and, therefore, do not fully explain the role of genetics in HEV infection. Wild turkeys have consistently tested negative for antibodies against HEV (Domermuth et al., 1977a, Hopkins et al., 1990). Host factors may play a greater role in the susceptibility of turkeys to HEV than previously thought. However, additional studies should be done with inbred lines of turkeys to explore more fully this aspect of HEV pathogenesis.

Chukar partridges, chickens, and peafowl have been experimentally infected with HEV (Domermuth and Gross, 1991, McFerran et al., 1997). However, death does not occur in non-target species infected with HEV. Antibodies to HEV have not been detected in the sera of 42 species of wild birds surveyed (Domermuth et al., 1977a).

2. Hemorrhagic enteritis virus infection of chickens.

Some reports indicate that leghorn strains of chickens are more susceptible to infection with HEV than are strains of broiler chickens (Beasley and Clifton, 1979). However, chickens inoculated with virulent HEV have not been reported to show any clinical signs of disease

independent of strain (Beasley and Clifton, 1979). Gross and histologic splenic lesions occurred in 20-40% of chickens experimentally inoculated with HEV (del Fierro, 1985). Spleens from infected birds were twice the size of spleens from uninoculated control birds. Histologically, intranuclear inclusions in lymphoreticular cells surrounding the sheathed arterioles of the white pulp, white pulp hyperplasia, and splenic lymphoid necrosis have been observed (del Fierro, 1985, Beasley and Clifton, 1979, Silim et al., 1979). Lymphoid hyperplasia in the GALT of the upper small intestine sometimes obliterating intestinal villi has been reported in experimentally infected chickens (Silim et al., 1979). Inclusions were observed in the large mononuclear cells in the lamina propria of intestines with lymphoid hyperplasia (Silim et al., 1979).

D. Immunity and protection.

The development of a detectable humoral immune response has good correlation with protection from HEV challenge. The role of cell mediated immunity is more poorly defined. CD4+ T-lymphocytes (helper T-lymphocytes) increase in the spleens of infected turkeys 4-6 days pi (Suresh and Sharma, 1995, Suresh, 1995). CD8+ suppressor T-lymphocytes also

increase in percentage post infection (Suresh and Sharma, 1995, Suresh, 1995). Depletion of T-lymphocytes with cyclosporin A enhances splenic lesion formation and viral replication in pheasants infected with MSDV (Fitzgerald et al., 1995).

The immunity induced by HEV is very long lasting. In one flock monitored over a 4 year period there was 100% seroconversion 4 weeks pi and was still at 83% positive after 40 months (McFerran et al., 1997). It is difficult to determine in cases such as the one reported, if the humoral immunity measured is due to the initial inoculation or due to reinfection. Since pathogenic and apathogenic HEVs are shed in the feces of infected turkeys and since HEV survives at 37 C for 4 weeks (McFerran et al., 1997), reinfection occurs readily in most turkey flocks after natural infection or vaccination.

Convalescent turkey serum administered to susceptible turkeys was the first method used to prevent outbreaks of HE (Domermuth et al., 1975). Gross lesions in the intestine and spleen could be prevented with 0.5-1.0 ml of convalescent serum and intestinal lesions could be prevented with 0.1-0.25 ml of convalescent serum (Domermuth and Gross, 1975). Hyperimmune anti-HEV turkey serum was shown to prevent HE for up to 5

weeks pi (Fadly and Nazerian, 1989). Later, turkey spleens with HEV and pheasant spleens with MSDV were processed, diluted 1:2 and administered to susceptible flocks in the drinking water (Domermuth et al., 1977b). Recent evidence suggests that MSDV, long considered apathogenic for turkeys, is immunosuppressive (Sharma et al., 1992, Sharma, 1994). The administration of the spleens of HEV inoculated turkeys to susceptible birds is also immunosuppressive and has the potential to introduce other problems as well.

A tissue culture attenuated HEV has been used extensively as a vaccine (Fadly et al., 1985). This vaccine is produced by passing virulent HEV in RP19 cells (Nazerian and Fadly, 1982, Fadly and Nazerian, 1984). The RP19 cell line is a Marek's disease virus (MDV) transformed turkey B-lymphocyte cell line which carries infectious MDV and can produce Marek's disease if inoculated into chickens (Nazerian et al., 1982). The tissue culture attenuated HEV vaccine has also been highly effective in preventing HE, although it too is immunosuppressive (Sharma, 1994).

Avirulent strains of HEV including MSDV have been proposed as vaccines. These non-pathogenic viruses have been grown in blood leukocytes (van den Hurk, 1990a, van den Hurk, 1990b).

Some new vaccination methods for HEV have been proposed in recent years. MSDV was successfully used to vaccinate SPF turkey poults at 24 days of embryonation. In ovo vaccinated poults were shown to be fully protected from challenge with 10^4 TCID virulent HEV at 4 weeks of age (Ahmad and Sharma, 1993). Additionally, the use of AASV has been proposed as a potential vaccine virus against HEV (Nagaraja et al., 1994). Finally, another tissue culture attenuated HEV vaccine is being developed which does not cause splenomegaly and therefore may not cause immunodepression (Sharma et al., 1995).

III. Molecular biology of adenoviruses

A. Genomic organization of adenoviruses

The organization of the adenoviral genome is highly conserved among mastadenoviruses (Sussenbach, 1984). The recently published CELO genome sequence shows that its genomic organization has several differences from the typical mastadenovirus organization (Cai and Weber, 1993, Chiocca et al., 1996). The central portion of the genome, where the structural protein genes are located, is conserved between CELO virus and the mastadenoviruses. The genes for the hexon, penton base, pIIIa, fiber,

pVI, pVII, pVIII, and the E2 region are present and in the same locations in the CELO virus genome as in mastadenoviral genomes (Chiocca et al., 1996). There is, however, 5 kb of sequence at the left end and 15 kb at the right end of the CELO virus genome with little or no sequence identity with mammalian adenoviruses. In addition, there are no E1, E3, and E4 regions identified in CELO virus. However, there are several open reading frames unique to CELO virus which are recognized at the left and right ends of the genome. One of these open reading frames (ORFs), ORF 8 or GAM-1, has been determined to share an anti-apoptotic function with the E1b 19kD protein and Bcl-2 (Chiocca et al., 1997). GAM-1 is located in the 15 kb of sequence unique to CELO at the right end of the genome. The virus associated (VA) RNA is found at the right end of the CELO virus genome (Larsson et al., 1986, Chiocca et al., 1996) and a dUTPase at the left end, opposite to mastadenoviruses (Chiocca et al., 1996). These changes have led to speculation that the CELO virus has undergone some rearrangement of the genome around the central block of structural genes in which the immortalizing and transforming genes of the E1 region have been moved to the left end of the genome and other genes to the right end of the genome (Chiocca et al., 1996). GAM-1 bears no DNA or amino acid sequence

similarity to the E1 region which carries the genes involved in immortalization and transformation in other adenoviruses.

In contrast to CELO virus, EDSV has most of the same transcription units described in mastadenoviruses in the same locations, although the E3 transcription unit has not been located and there are several ORFs at the right end of the genome to which no function has been assigned (Brandt et al., 1997). In the information available on the genomic organization of HEV, the E1b region, penton base, pVI, and core protein genes are all in the same locations as they are in mastadenoviruses (McQuiston et al., 1995). Although the information is sparse, the presence of an E1b transcription unit near the left end of the genome, suggests that HEV has not undergone the same rearrangement of the genome seen in CELO virus. Some authors have speculated that HEV has undergone significant genomic rearrangements in comparison to mastadenoviruses (Jucker et al., 1996). However, this conclusion is not supported by published data.

Before sequencing was available as a research tool, aviadenoviruses and mastadenoviruses were compared with a variety of other techniques. Using a hybridization technique, several oncogenic and non-oncogenic human adenoviruses were compared. DNA heteroduplexes were formed

between strands of DNA from different serotypes in the region of the hexon (Garon et al., 1973). In other hybridization experiments, human adenovirus type 2 (Ad2) and CELO virus were compared. Two regions were found to hybridize under stringent conditions. The areas of similarity were between map units 18.1 and 21.6 and between 57 and 58.5. The leftmost region of homology corresponds to the major late promoter and the rightmost region corresponds to the hexon gene (Alestrom et al., 1982a). Similarly, Larsen et al. (1979) found only two regions of homology between Ad2 and murine adenovirus FL. One region corresponded to the major late promoter (12-18 map units) and one corresponded to the hexon (51-62 map units) (Larsen et al., 1979). In contrast, a similar study done comparing Ad2 and bovine adenovirus type 3 (BAV 3) found that there were significant areas of hybridization between the two viruses corresponding to the areas between map units 10 and 80. These regions include the major late promoter and the late transcription units which encode the structural genes. The predicted hexon amino acid sequence of BAV 3 was compared to that of the Ad2 hexon and was found to be 70-80% identical. (Hu et al., 1984)

Sequence identity and similarity has also been detected in the internal terminal repeat (ITR) regions. The CELO virus ITR was compared to Ad5,

Ad3, Ad12, simian adenovirus type 7, and murine adenovirus FL ITRs. There is a common sequence between base pairs (bp) 9 and 14, (TA)ATAATA which may be a recognition sequence. It resembles a TATA box usually located adjacent to RNA polymerase II start sites (Alestrom et al., 1982b). The CELO virus ITR is 63 amino acids shorter than mastadenovirus ITRs. Additionally, the CELO virus ITR ends in a dGMP residue compared to the dCMP residue which ends the ITR of mastadenoviruses (Alestrom et al., 1982b).

B. Infectious cycle

Once the virus is attached to the cell surface, the process of penetration begins. Adenoviruses enter the host cell by receptor-mediated endocytosis, penetrate the cytosol from endosomes and deliver their DNA genome into the nucleus (Pombo et al., 1994).

In the host cell nucleus, viral RNA is transcribed from five regions of the viral DNA, and translated into 12 or more early proteins. Viral DNA replication proceeds from both ends by a strand displacement mechanism. Following DNA replication, mRNAs are transcribed from the late transcription units and translated into structural proteins (Fenner et al.,

1987). These late mRNAs are transcribed and translated in excess (Franklin et al., 1971). Virions are assembled in the host cell nucleus where they form the classic crystalline array. The virions are released via cell lysis (Philipson, 1983, Fenner et al., 1987, Cotran et al., 1989).

C. Virus attachment and entry

Infective virions attach to cellular surface receptors. In HEV, as with other adenoviruses, the fiber protein of the viral capsid binds with host cell receptors to initiate viral attachment (Fenner et al., 1987, Mei and Wadell, 1993). After attachment to cells, Ad2, Ad3, Ad4, and Ad12 bind to the surface of cells via αv integrins with an arginine-glycine-aspartic acid (RGD) sequence in the penton base polypeptide (Wickham et al., 1993). The penton base of type II aviadenoviruses lacks this RGD motif but does have a leucine-valine-aspartic acid (LVD) motif which is an essential sequence for the recognition of fibronectin by the α4β1 integrin receptor and may have a similar role for the penton base. The expression of α4β1 integrins is limited to the surfaces of immune system cells. The penton base may interact with α4β1 integrins on immune cells to mediate HEV entrance into host cells (Suresh, 1995). The FAV 10 penton base lacks both the RGD

and LVD motifs (Sheppard and Trist, 1992).

The penton base plays a crucial role in virus escape from endosomes. The penton base undergoes a pH dependent conformational change. This change increases the hydrophobicity of the penton base as the pH drops below 5. The hydrophobic penton base then interacts with and penetrates the lipid bilayer of the endosome (Seth, 1994, Cotten et al., 1993).

D. Transcription

Adenoviral transcription is summarized in Figure 1.

The early phase of transcription is usually in the first 3-5 hours of infection, before viral DNA replication begins (Bridge and Pettersson, 1996). Six regions of the adenoviral genome are transcribed early: the E1a, E1b, E2 (a and b), E3, E4, and L1 transcription units (Bridge and Pettersson, 1996, Lutz and Kedinger, 1996). Each early transcriptional unit has its own promoter (Berk and Sharp, 1977), and produces a single precursor RNA. The major late promoter (MLP) is active in the early phase, but only the L1 transcription unit is expressed (Bridge and Pettersson, 1996). Proteins which act to restrict cell growth and protein required for DNA replication are expressed in the early phase (Pombo et al., 1994).

Adenoviral DNA is transcribed in both early and late phases by host RNA polymerases I and II. Transcription is predominantly detected in sites in the nucleus which are separate from the sites of DNA replication (Pombo et al., 1994).

After the early phase, the intermediate genes are transcribed. IVa2 and IX are transcribed at the beginning of DNA synthesis. Following the intermediate phase, the MLP is activated. Two factors, DEF-A and DEF-B add to MLP activation by cooperatively binding to downstream elements which form a downstream control region of the MLP. DEF-B is the protein product of IVa2 (pIVa2). This protein, while monomeric in solution binds as a dimer to the downstream control region of the MLP. DEF-A also binds to this control region. DEF-A may be a heterodimer of pIVa2 and a 40 kD unknown polypeptide (Lutz and Kedinger, 1996).

The late mRNAs which are initiated from the major late promoter (MLP) have a 200 bp leader sequence derived from the tripartite leader sequence transcribed from map units 16, 19, and 26 (Nevins and Darnell, 1978, Anderson and Lewis, 1980, Miller et al., 1980). A common run on precursor RNA is transcribed and subsequently processed into approximately 20 late mRNAs (Miller et al., 1980). Late phase splicing

takes place in clusters of small nuclear ribonucleoproteins separate from sites of DNA replication (Bridge et al., 1995). The switch from early to late gene expression requires the replication of the viral DNA template (Lutz and Kedinger, 1996). There are data which suggest that the late RNA precursor is spliced during and immediately after transcription (Bridge and Pettersson, 1996, Pombo et al., 1994). Capping, polyadenylation, and methylation of viral mRNAs is carried out by the host cell's machinery (Pombo et al., 1994).

E. DNA replication

Adenoviral DNA replication may begin at either end of the linear dsDNA genome. The origin of replication lies within the internal terminal repeat (ITR) at the ends of the genome. It appears that 20 bp in the ITR are essential for the initiation of replication. The ITR has two distinct regions: an AT rich region of 50-52 bp at the end of the genome and 50-110 bp of a GC rich region adjacent to the AT rich region. The AT rich portion of the ITR may function in local melting during DNA replication. The sequence of the ITR is highly conserved among adenoviruses (Tamanoi and Stillman, 1983).

Two viral and several host proteins are required for the initiation of DNA replication. They are the terminal protein (TP; located in the E2b transcription unit), DNA polymerase (located in the E2b transcription unit), the host transcription factors NF-1/CTF and NF-III/OTF1, and a host protein with topoisomerase activity (Pombo et al., 1994). A DNA binding protein (DBP; located in the E2a transcription unit) is also required for chain elongation (Sussenbach and van der Vliet, 1983).

Each 5' end of the genome is bound covalently with a phosphodiester bond to the TP forming a pTP-dCMP complex. The adenoviral DNA polymerase is required to make this complex. The pTP and DNA polymerase form a complex which recognizes a 9-22 bp sequence in the adenovirus template strand of DNA. The TP is associated with the DNA polymerase which is, in turn, complexed with the genomic DNA (Pronk et al., 1992). Newly synthesized pTP is 82 kD and is cleaved by a 23 kD adenoviral protease to the mature TP (55 kD) late in infection. Anti-TP and anti-pTP antibodies block both initiation and chain elongation by inhibiting the formation of the (p)TP-DNA complex (Tamanoi and Stillman, 1983).

Chain elongation requires a DBP encoded in the E2a transcription unit. The carboxy terminal end of the adenoviral DBP binds ssDNA

(Brough et al., 1993). DNA replication is by displacement strand synthesis. The displaced strand becomes a daughter by complementary strand synthesis.

One of the features of adenovirus infection is the shut down of the host cell metabolism. During the intermediate and late phases, cellular genes are transcribed and processed but are no longer transported to the cytoplasm. The result is preferential export of viral RNAs. Additionally, viral RNAs are preferentially translated over host mRNAs in the cytoplasm (Bridge and Pettersson, 1996).

F. Early transcription units

1. E1 transcription unit

The E1 region is usually deleted in the replication defective adenoviruses used as vectors (Graham, 1990, Gorziglia et al., 1996). The transforming region of mastadenoviruses, E1, is in the carboxy terminal 11-12%. The evidence for the E1 region as transforming, comes from the demonstration of transformation with restriction fragments from this region and by analysis of viral RNA transcripts from adenovirus transformed cell lines and the abrogation of oncogenicity by deletion of the E1 region

(Subramanian et al., 1993). The E1a region is transcribed from the rightward transcribed strand of the adenovirus genome (r-strand), between map units 1.3 and 4.6 (Sussenbach, 1984). The E1a transcription unit encodes factors which regulate the expression of adenovirus early genes (Bridge et al., 1991). E1a induces aneuploidy and immortality in cells *in vitro* (Lowe and Ruley, 1993). Five proteins are encoded by the E1a region. The E1b region is transcribed from the r-strand, between map units 4.6 and 11.2 (Petterson et al., 1983, Sussenbach, 1984). The E1b region encodes three proteins involved in transformation, including altered cellular morphology, rapid growth, tumorigenicity, and loss of contact inhibition (Green et al., 1983, Sussenbach, 1984, Quinlin, 1993). During lytic infection, E1b proteins are involved in DNA replication (Sussenbach, 1984).

2. E2 transcription unit

E2a encodes the ssDNA DBP required for DNA chain elongation during replication. E2b encodes two proteins: a primer protein and the terminal protein precursor (pTP) (Sussenbach, 1984).

3. E3 transcription unit

The E3 transcription unit is non-essential *in vitro* and is often replaced by foreign DNA in adenovirus vector systems (Graham, 1990, Doronin et al., 1993, Gorziglia et al., 1996). The E3 region plays a role in the evasion of the host immune response by adenoviruses such as the reduction in the expression of the major histocompatibility complex class I (Ginsberg et al., 1989, Routes and Cook, 1990, Gooding, 1992, Hermiston et al., 1993). The 3' portion of E3 encodes the 10.4 kD, 7.5 kD, 14.5 kD, and 14.7 kD proteins which change the nature of the inflammatory response (Ginsberg et al., 1989). The 14.7 kD alone and the 14.5 kD together with the 10.4 kD protein protect cells from lysis by TNF (Tufariello et al., 1994) . The 14.5 kD/10.4 kD complex down regulates expression of the epidermal growth factor (EGF) receptor (Carlin et al., 1989; Tufariello et al., 1994). The mechanism by which these proteins exert their effects is not clear.

4. E4 transcription unit

The products of the E4 transcriptional unit function in post-transcriptional events in viral late gene expression and in transcriptional

regulation of E2. E4 products may also play a role in the regulation of viral DNA regulation (Bridge et al., 1991, Bridge et al., 1993).

G. Late transcription units

The late transcription units are transcribed from the r-strand of the genome between map units 31.0 and 91.3 (Sussenbach, 1984). Primarily the structural protein genes of the adenovirus virion are transcribed from the late transcription units.

H. Adenovirus virion

The capsid of adenoviruses is icosahedral and is composed of 252 capsomeres, 240 of which are hexons and 12 of which are pentons (Philipson et al., 1975, van Oostrum and Burnett, 1985). There are 180 hexons which make up the 20 triangular faces of the icosahedron and 60 total peripentonal hexons which surround the pentons at the twelve vertices. The pentons consist of a penton base with one or more attached fibers (Philipson et al., 1975, Philipson, 1983, Sussenbach, 1984). The icosahedral capsid covers a core containing a complex of DNA and proteins.

1. Core proteins

The core was first identified with electron microscopy. It is a compact structure, 34 nm in diameter, with morphology similar to a chromatin fiber. The nucleoprotein contains the pVII, pV, and μ proteins. All three core protein genes are in the L2 transcription unit (Alestrom et al., 1984). Purified pVII forms a stable complex with DNA protecting 100-150 bp of DNA in a manner similar to histones. The pV protein forms a shell around the nucleoprotein complex (Philipson, 1983, Sussenbach, 1984).

2. Capsid proteins:

a. Hexon

The hexon, found in the adenoviral capsid, is a trimer (Grütter and Franklin, 1974, van Oostrum and Burnett, 1985) composed of stable but non-covalently associated hexon polypeptides (Cepko and Sharp, 1983, Cornick et al., 1973). The trimeric, native hexon is recognized by different antibodies than is the nascent hexon (Cepko et al., 1981, Fortsas et al., 1994). From this point, hexon will refer to the native, trimeric hexon and the denatured, monomeric, nascent hexon polypeptides will be indicated as

such. Hexons represent the dominant viral protein both in the virion and in the infected cell and are, therefore, the major antigenic component (Monreal, 1992).

Early descriptions of the hexon were of a solid sphere (Horne et al., 1959, Valentine and Pereira, 1965). Later, the hexon was described as a hollow sphere or polygon (Wilcox and Ginsberg, 1963, Petterson et al, 1967). More recent reports show the hexon has a threefold symmetry based on electron microscopy and crystal structure. The hexon consists of two structural parts including a triangular top 64 angstroms tall with three towers and a pseudo-hexagonal base 52 angstroms tall with a central cavity (Athapilly et al., 1994, Roberts et al., 1986). The lowest 1 nm of the hexon facing the DNA core, is 7.5 nm in diameter with an axial hole 3.5 nm in diameter. The mid 1-5.2 nm is hexagonal with an 8.9 nm side while the top 5.2-11.6 nm is triangular with a 7.5 nm side (Philipson, 1983). The internal surface of the hexon is hydrophobic while the external surface is negatively charged (Philipson, 1983). From the pseudo-hexagonal symmetry of the base arises two kinds of vertical hexon to hexon contact faces which alternate around the base. These are the A face, under each tower, and the B face, lying between the towers (Roberts et al., 1986). Each contact is A face

to B face between hexon subunits in the viral capsid (Roberts et al., 1986). The three identical hexon polypeptides are tightly interwoven where they interface. Each tower is formed from three loops, one from each hexon polypeptide (Roberts et al., 1986, Athapilly et al., 1994).

The hexon gene of adenoviruses is located in the L3 transcription unit (Mautner et al., 1975, Lewis et al., 1975, Lewis et al., 1977, Sussenbach, 1984) and the translated protein is 966 amino acids long in Ad2. The entire hexon transcript is translated (Jornvall et al., 1981b). The primary structure of the hexon polypeptide has some unique features. The hexon polypeptide is acidic with an excess eleven acidic residues over the sum of basic residues in Ad2. The CELO virus hexon is highly acidic containing 26% aspartic and glutamic acid residues (Laver et al., 1971). Nine charged residues cluster to form a highly acidic region on the hexon surface (Philipson, 1983). In the hexon polypeptide, prolines are common in the first 335 amino acids (27/335; 8.1%), uncommon in next 333 amino acids (10/333; 3%), and common in the last 298 amino acids (20/298; 6.7%) in Ad2 (Jornvall et al., 1981b). The amino terminus of the hexon polypeptide is acetylated. Secondary structure is limited in the hexon polypeptide with 8% in α helix and 22% in β pleated sheet (Roberts et al., 1986). The

primary and secondary structure of the hexon polypeptide are highly conserved (Franklin et al., 1971, Kinloch et al., 1984, Toogood et al., 1989).

b. 100 kD folding protein

The 100 kD folding protein gene is approximately 2.3 kb in Ad2 and located in the L4 transcription unit (Lewis et al., 1975, Lewis et al., 1977, Sussenbach, 1984). The protein is post translationally processed into a phosphoprotein (Cepko and Sharp, 1982). The 100 kD folding protein has roles in the formation of the native hexon and in the efficient translation of late adenoviral mRNAs. The 100 kD folding protein can bind to cytoplasmic mRNA (Adam and Dreyfuss, 1987, Riley and Flint, 1993). A link between the ability of the 100 kD folding protein to bind to mRNA and its ability to facilitate the translation of that mRNA has not been established. However, the selective binding of mRNAs by 100 kD folding protein in the late phase of infection may lead to the selective translation of the late phase viral mRNAs. One candidate sequence for recognition of the late mRNAs by the 100 kD folding protein is the tripartite leader sequence. The primary role of the 100 kD folding protein may be to direct viral late

mRNA species to, or keep them in, a cytoplasmic compartment in which their translation is facilitated (Hayes et al., 1990)

c. Hexon folding

The nascent hexon requires the co-expression of the 100 kD folding protein to realize the complex configuration of the hexon. The 100 kD folding protein plays roles in the formation of hexon trimers (Morin and Boulanger, 1986) and in the transport of the hexon trimers to the nucleus (Gambke and Deppert, 1983, Cepko and Sharp, 1983, Oosterom-Dragon and Ginsberg, 1981, Williams and Ustacelebi, 1971). Much of the work on the nature of the 100 kD folding protein and hexon polypeptide interaction has been done using temperature sensitive (ts) adenovirus mutants (Grodzicker et al., 1977, Cepko and Sharp, 1983, Young et al., 1984). Two types of ts mutants in Ad5 have been defined: "hexon minus" mutants (Russell et al., 1972, Leibowitz and Horwitz, 1975) which fail to produce hexons at non-permissive temperatures and "transport" mutants (Russell et al., 1972, Kauffman and Ginsberg, 1976) which produce hexon trimers which are not transported to the nucleus. The hexon minus mutants have mutations in the hexon gene while the transport mutants have mutations

which map to the L4 transcription unit, specifically to the 100 kD folding protein gene (Williams and Ustacelebi, 1971, Williams et al., 1974).

The 100 kD folding protein interacts with the hexon mature protein as well as with complete, newly synthesized hexon polypeptides but is not found in the mature virion (Grütter and Franklin, 1974, Cepko and Sharp, 1983, van Oostrum and Burnett, 1985). Virtually all of the hexon polypeptide bound to the 100 kD folding protein is destroyed by trypsin and therefore not in the native conformation (Cepko and Sharp, 1982). The complex of hexon polypeptide and 100 kD folding protein is approximately 800 kD and 1,000 kD species exist. The majority of the 100 kD folding protein is found in 800 kD complexes with the hexon polypeptide. The hexon polypeptide and 100 kD folding protein transiently associate on the polyribosomes during translation and remain as a complex in the cytoplasm (Cepko and Sharp, 1982). This complex is located in the cytoplasm primarily, although some can also be found in the nucleus. Pulse-chase experiments in concert with immunoprecipitations with anti-hexon and anti-100 kD folding protein monoclonal antibodies, revealed that the hexon is formed at the time when the hexon polypeptides are released from the 800 kD complex. The 100 kD folding protein-hexon polypeptide complex thus

plays a major role in hexon assembly and may actually direct the folding of the hexon monomers into the trimeric, native conformation (Cepko and Sharp, 1983).

d. Penton

The penton forms the 12 vertices of the adenoviral capsid (Philipson et al., 1975, Philipson, 1983, Sussenbach, 1984). The amino terminal 20 amino acids of the fiber are joined non-covalently to the penton base to form the penton (Henry et al., 1994). The penton is composed of two penton base monomers and three fiber monomers (van Oostrum and Burnett, 1985). Penton bases and fibers readily assemble *in vitro* with no additional proteins required. A recombinant baculovirus system has been used to express the penton *in vitro* (Novelli and Boulanger, 1991a, Novelli and Boulanger, 1991b). The penton base and fiber are synthesized on different polyribosomes and within minutes the fiber and penton base subunits accumulate and are assembled into pentons. (Monreal, 1992, Philipson, 1983).

The penton base gene is located in the L2 transcription unit (Lewis et al., 1975, Lewis et al., 1977, Sussenbach, 1984). Some of the cytopathic

effect of adenoviruses has been attributed to the penton base (Pereira, 1958, Everett and Ginsberg, 1958). The RGD motif in the penton base mediates the cytopathic effect associated with the purified protein (Bai et al., 1993).

e. Fiber

The fiber gene is located in the L5 transcription unit (Mautner et al., 1975, Lewis et al., 1975, Lewis et al., 1977, Sussenbach, 1984). The fiber is a glycoprotein composed of three 62 kD polypeptides which form a long structure with a terminal knob. The diameter of the rod portion of the fiber is 2 nm and the diameter of the knob is 4 nm. Most mastadenoviruses have a single straight fiber. Ad40, however, has two fibers of differing lengths. Each penton, in the case of Ad40 has only one fiber (Kidd et al., 1993). In contrast, FAVs have two fibers in each penton. The length of type I aviadenoviruses fibers differs between serotypes. The fiber pairs of FAV2 - 11 are of similar size, 22-28 nm. CELO virus (FAV 1), however, has one long (46 nm) and one short (11 nm) fiber. The fibers are flexible and lay at diverse angles in the viral capsid. The second, shorter fiber of CELO virus lacks a knob element (Monreal, 1992). Similar to the FAVs, BAV 3 pentons each have a single, long fiber which is bent along the shaft

(Ruigrok et al., 1994). The type II aviadenoviruses have a single, short fiber (van den Hurk, 1992). Some reports suggest that MSDV and AASV lack fibers completely (Zhang et al., 1991).

Anti-fiber antibodies prevent the attachment of adenoviruses to erythrocytes, thereby preventing hemagglutination. There are 10^4 fiber receptors per red blood cell. The knob portion of the fiber interacts with cellular receptors (Henry et al., 1994). Most nucleotide and amino acid changes between human adenovirus serotypes lie in the knob region of the fiber (Eiz et al., 1995).

f. Other capsid proteins

pIIIa is an internal capsid protein. It forms a bridge between pentons and peripentonal hexons (Stewart et al., 1991, Stewart et al., 1993).

pVIII binds the nucleoprotein core to the internal surface of the capsid (Stewart et al., 1991, Stewart et al., 1993).

pVI is the hexon associated protein. It binds to the nucleoprotein core and connects the core to the ring of peripentonal hexons (Stewart et al., 1991, Stewart et al., 1993, Matthews and Russell, 1994).

pIX is expressed intermediate and late in infection (Philipson, 1983). The hexons of the 20 regular, triangular faces of the adenoviral virion are associated as groups of nine, connected by pIX located on the internal surface of the capsid (Philipson, 1983, Furcinetti et al., 1989). In human enteric adenoviruses (Ad40, Ad41, Ad31, Ad3, and Ad7), hexon trimers predominate after gentle dissociation of the viral capsid. In human respiratory adenoviruses (Ad2 and Ad5), groups of nine and higher order hexons predominate after dissociation (Fortsas et al., 1994). Groups of nine could not be produced from CELO virus (Laver et al., 1971). Recent sequence analysis of CELO virus confirms the lack of a pIX gene in this virus (Chiocca et al., 1996). The biological significance of these findings is not yet known. Mutant virions which lack pIX have a maximum capacity for DNA approximately 2 kb less than the normal length of the adenoviral genome (Ghoush-Choudhury et al., 1987).

g. Proteins of type II aviadenoviruses

The polypeptides of the three type II aviadenoviruses, HEV, MSDV, and AASV have been characterized with Western blots and immunoprecipitation techniques using both monoclonal antibodies and

polyclonal antibodies. With polyclonal anti-HEV antibodies, 12 polypeptides have been described in virulent HEV. The sizes of the polypeptides are approximately 96-97 kD (pII, hexon), 57-55 kD (pIII, penton base), 51-45 kD (pIVa, fiber), 44 kD, 37-43 kD 9 (pV, core protein), 34 kD, 25-29 kD (pVI, hexon associated protein), 20-24 kD, 19.5-21 kD, 19 kD (pVII, core protein), 12.5-14.5 kD, and 9.5 kD (Nazerian et al., 1991, van den Hurk, 1992, Zhang et al., 1991). Avirulent HEV was reported to have polypeptides of the same size as virulent HEV with the exception of the penton base which appeared to be 51 kD in virulent HEV while it appeared to be 52 kD in avirulent strains in one report (van den Hurk, 1992). In another report, the polypeptides of the three type II avian adenoviruses were compared and found to be identical with the exception of the fiber which was not found in MSDV and AASV. This finding was confirmed with electron microscopy in which the viruses appeared to be indistinguishable except that no fibers were observed at the vertices of MSDV and AASV capsids (Zhang et al., 1991). Fiber protein is the most variable adenovirus component both in size and antigenicity. There has been speculation that these differences in penton base and fiber could explain the difference in pathogenicity between virulent and avirulent

strains of HEV (van den Hurk, 1992). Sequence comparisons of the penton base genes of virulent HEV and MSDV show they are 100% identical (Suresh, 1995).

3. Assembly of capsids

Late in adenoviral infection, viral polypeptides are rapidly released from polyribosomes and transported to the nucleus (Philipson, 1983). Shortly after translation, the monomeric subunits of the structural polypeptides assemble into the multimeric proteins of the capsid. The penton assembles rapidly for the first 25% of newly synthesized polypeptides but takes nearly 24 hours to be completed. Native hexon accumulated in the nuclei of infected cells within 5 min. of dissociation from polyribosomes.

Empty capsids assemble in the host cell nucleus. Mature virions are formed by the insertion of viral DNA and core proteins into these preassembled empty capsids (Philipson, 1983). Only about 10% of the total viral DNA is packaged into virions. There is a packaging signal which lies between 290-390 nucleotides (nts) from the left end of the viral genome and which is essential for the packaging of viral DNA (Philipson, 1983).

IV. Immunogenicity of adenoviruses

The precise mechanism by which antibodies neutralize adenoviruses has not been determined. There are three structural proteins to which antibodies can bind and thereby inactivate infectivity: the fiber, the penton base and the hexon. Anti-fiber antibodies cause the aggregation of virions. Anti-penton antibodies prevent the release of the virus from the endosome after virus entry into the host cell (Varga et al., 1990). Anti-hexon antibodies may act by both aggregation of virions and by the inhibition of conformational change in the hexon in the acid endosome. This conformational change is essential to virus escape from the endosome (Varga et al., 1990).

The α antigen of adenoviruses is associated with the internal surface of the hexon, except in bovine and CELO virus which lack this antigenic determinant (Monreal, 1992). Hexons also carry the ε antigenic determinant, the type specific antigen, on the external surface of the capsid (Norrby and Wadell, 1969, Willcox and Mautner, 1976a, Willcox and Mautner, 1976b, Toogood et al., 1992). Seven hypervariable regions differing in both length and sequence were found which correspond to type

specificity (Crawford-Miksza and Schnurr, 1996). Five antigenic epitopes have been identified associated with the hexon using monoclonal antibodies (Adam et al., 1987, Monreal, 1992). These epitopes are grouped into three antigenic clusters (Adam et al., 1987).

The fiber carries one type specific determinant, γ, which is in the knob region (Norrby and Wadell, 1969). Human adenoviruses with short fibers (subgroup B), have only the γ determinant. Adenoviruses with longer fibers also have a δ determinant located at the junction of the fiber and the penton base. The δ determinant is masked in the intact penton. The penton base carries the β antigenic determinant (Wadell and Norrby, 1969). The antigenic determinants of adenoviruses are summarized in Table 1.

Hexon monoclonal antibodies are neutralizing for HEV and MSDV *in vitro* (Nazerian et al., 1991, van den Hurk and van Drunen Littel-van den Hurk, 1993). Additionally, hexon monoclonal antibodies inoculated into 6 week old turkeys protected them from challenge with virulent HEV. Turkeys inoculated with native hexon protein were protected from the lesions of HE and HEV infection after challenge with virulent HEV. In contrast, turkeys inoculated with denatured hexon were not protected when challenged with virulent HEV (van den Hurk and van Drunen Littel-van den

Hurk, 1993).

In mice inoculated with replication defective adenovirus vectors, anti-hexon antibodies appeared first, at day 26 pi followed by anti-fiber antibodies on day 35 pi and finally by anti-penton base on day 45 pi (Gahery-Segard et al., 1997). These results probably reflect the percentage of the adenovirus virion composed of the capsid proteins. The hexon composes 60% of the capsid (Monreal, 1992) and the penton a much smaller percentage.

V. Poxviruses

Poxviruses are divided into Chordopoxviridae and Entomopoxviridae based on a vertebrate or invertebrate host range, respectively (Moss, 1985, Moss, 1992, Buller and Palumbo, 1991). Chordopoxviruses share a group specific nucleoprotein precipitinogen (Buller and Palumbo, 1991). Chordopoxviruses are further divided into several genera: orthopoxvirus (prototype virus: vaccinia), parapoxvirus (prototype virus: orf), avipoxvirus (prototype virus: fowlpox virus {FPV}), capripoxvirus (prototype virus: goat pox), leporipoxvirus (prototype virus: myxoma), suipoxvirus (prototype virus: swine pox), and the unclassified poxviruses

(Moss, 1985). FPV is the prototype virus of the avipoxvirus, a genus which contains a number of antigenically distinct but related viruses that infect birds and are of considerable commercial importance. FPV has a worldwide distribution and its natural host is the chicken (Buller and Palumbo, 1991).

A. Molecular biology of poxviruses

The poxviruses are 200-400 nm in length with an axial ratio of 1.2 to 1.7 (Moss, 1992, Moss, 1985, Buller and Palumbo, 1991). Entomopoxviruses are kidney shaped with a single lateral body (Moss, 1985). Chordopoxviruses are oval or brick-shaped with two lateral bodies in the bilateral concavities of the core (Moss, 1992, Buller and Palumbo, 1991). The core contains a twisted and folded nucleoprotein fiber (Moss, 1985, Moss, 1992). A 50-55 nm lipoprotein bilayer membrane surrounds the core. The outer surface of the membrane has a textured appearance due to randomly arranged tubule elements. The lipid composition of the membrane is distinct from host lipid bilayers unlike other enveloped viruses (Buller and Palumbo, 1991).

The intracellular naked virion (INV) composed of the nucleoprotein core and lateral bodies surrounded by a membrane, is infectious.

Extracellular enveloped virions (EEV) have, in addition to the structures of the INV, a lipoprotein envelope with at least seven glycoproteins. INVs are harvested from infected cells while EEVs are harvested from media (Moss, 1992). Entry by EEV into the host cell is faster than INV entry (Moss, 1992). Chicks given FPV INV alone and FPV INV+EEV developed similar levels of anti-FPV humoral immunity. However, since the EEV given was at much lower titer, the conclusion is that EEVs are more immunogenic than INVs (Saini et al, 1990).

The poxvirus genome is linear, AT rich, dsDNA, and 130-300 kb in length (Moss, 1992, Moss, 1985, Buller and Palumbo, 1991). FPV has a genome of 254-300 kb (Tripathy and Reed, 1997). The poxvirus genome is characterized by an absence of introns, short promoters, and small ORFs. The 189 kb vaccinia genome encodes more than 200 genes. Non-essential genes are clustered near the ends of genome (Moss, 1992). Recombination occurs at a high rate in the terminal regions of the poxvirus genome (Moss, 1992, Buller and Palumbo, 1991).

B. Infectious cycle

The poxvirus infectious cycle occurs in the cytoplasm. In vaccinia

virus, the cycle takes between 35 and 75 hours for maximum levels of progeny to be produced (Buller and Palumbo, 1991). FPV replication in chicken dermis produces infectious virus 72-96 hours pi (Tripathy and Reed, 1997). The poxvirus virion fuses with the cell in a pH independent manner. The fusion is much more rapid for EEV than for INV. Electron microscopy shows that INVs enter the cell by surface fusion and endocytosis (Moss, 1992). Vaccinia virus entry into cells can be blocked by monoclonal antibodies against any of five polypeptides in the virion membrane or by mouse or rabbit anti-vaccinia polyclonal antisera (Moss, 1992).

After fusion, the first uncoating, begins with the viral core being injected into the cytoplasm of the host cell (Buller and Palumbo, 1991, Beaud, 1995).

The next step in infection involves the transcription of early genes. Regulatory sequences for the initiation of transcription of early genes lie upstream of RNA start sites (Buller and Palumbo, 1991, Moss, 1992). Poxvirus promotors are approximately 30 bp long, have early and/or late activity, and can function equally in most poxviruses (Boyle and Coupar, 1986, Boyle, 1992). In genes transcribed before DNA replication,

termination occurs 20-50 bp downstream from any TTTTTNT sequence (Yuen and Moss, 1987, Shuman et al., 1987). Enzymes transcribed early in poxvirus infections include RNA polymerase, a transcription factor, capping and methylating enzymes, a termination factor, poly A polymerase, and a topoisomerase. Early mRNAs have typical eukaryotic characteristics but late mRNAs are long and heterogeneous appearing as smears due to the lack of a common termination sequence (Moss, 1992).

After early gene transcription, the second uncoating begins. The second virus uncoating involves the removal of the proteins of the nucleoprotein core. At this stage, the genome becomes DNase sensitive (Buller and Palumbo, 1991, Beaud, 1995).

The next step is the expression of late genes. Late gene expression requires at least three intermediate regulatory genes. The late poxvirus transcripts differ from early transcripts in the following ways: 1) Late promoters contain a TAAAT sequence within which transcription initiates, and 2) There are no termination signals at the 3' ends of the late genes (Moss, 1992). Early protein synthesis ceases with the onset of late protein synthesis unless the promoter has both early and late activity as do most promoters used to express foreign genes.

Poxvirus replication occurs in discrete areas of the cytoplasm called factories or viroplasm (Buller and Palumbo, 1991). Viral proteins are predominantly or exclusively used for viral DNA replication (Moss, 1992, Beaud, 1995). DNA replication can be detected within two hours of infection (Moss, 1992). Replication of FPV in chicken dermal epithelium begins between 12 and 24 hours pi (Tripathy and Reed, 1997).

Poxviruses replicate using a self-priming model of replication. Terminal hairpins lie at the ends of the poxvirus genome making the dsDNA genome into a continuous strand (Moss, 1992, Beaud, 1995). During replication, a nick is introduced near the 3' end that can be extended to form a palindrome which then folds back on itself to replicate the remainder of the genome. Replication begins at one or both ends of the genome. There is no specific origin of replication in poxviruses (Moss, 1992). Large concatemeric species are generated during poxvirus replication. These concatemers are resolved into mature DNA molecules and incorporated into virions late in infection (Beaud, 1995).

Poxvirus proteins are transported in association with actin filaments to the cell periphery where they are enveloped by membranes derived from the Golgi apparatus. Virions fuse with the plasma membrane to form EEV.

Expression of a viral 14 kD protein is required for the egress of virions from the host cell (Moss, 1992).

C. Poxvirus vectors.

Poxviruses have been widely used as vectors for foreign genes. Vaccinia virus was the first, still the most widely used, and most fully characterized poxvirus vector (Guo et al., 1990, Taylor et al., 1991a, Smith et al., 1992, Tartaglia et al., 1992, Alkhatib et al., 1994, Paoletti et al., 1994). Up to 25 kb of foreign DNA can be inserted into vaccinia vectors (Smith et al., 1992). Avipoxviruses have also been used extensively as vectors (Boursnell, 1992). FPV (Boyle and Coupar, 1988, Taylor et al., 1990, Ogawa et al., 1990, Webster et al., 1991, Yanagida et al., 1992, Nazerian et al., 1992, Calvert et al., 1993, Yoshida et al., 1994, Webster et al., 1996), and canary poxvirus (Taylor et al., 1991b, Cadoz et al., 1992, Taylor et al., 1992, Tartaglia et al., 1993, Taylor et al., 1994, Taylor et al., 1995, Fries et al., 1996) have been used as vectors in both avian and mammalian species (Taylor et al., 1992, Tartaglia et al., 1993, Taylor et al., 1994, Taylor et al., 1995, Fries et al., 1996). The thymidine kinase gene is the most frequently used site of insertion of foreign genes for vaccinia

(Gillard et al., 1985) and FPV recombinants (Boyle and Coupar, 1988, Taylor et al., 1988, Schnitzlein and Tripathy, 1990). Another undisclosed site near the FPV terminus has also been used for the insertion of foreign genes (Yanagida et al., 1992, Nazerian et al, 1992, Calvert et al., 1993, Yoshida et al., 1994). A vaccinia virus promoter, with both early and late activity, P7.5, is often used to drive the expression of foreign genes in vaccinia vectors (Smith et al., 1992). P7.5 has also been used in FPV vectors (Boursnell, 1992, Prideaux et al., 1990; Schnitzlein and Tripathy, 1990). Synthetic promoters with both early and late activity have been constructed for use in FPV vectors (Yanagida et al., 1992). Some of these synthetic promoters have greater activity than P7.5 in the FPV system (Calvert et al., 1993).

The infectivity and immunogenicity of vaccinia virus is dependent on route of inoculation (Andrew et al., 1992). Similarly, chicks inoculated intradermally with FPV were protected against challenge, while aerosol and drinking water inoculated chicks were not. Additionally, intradermal inoculation produced a longer period of FPV replication than did intratracheal inoculation. Chicks vaccinated with FPV in drinking water were 50% protected against challenge with wild type FPV. Additionally,

these chicks did not have long lasting protection with no immunity detectable at 92 days pi (Saini et al., 1990). Increased FPV titers of 10^6 plaque forming units (pfu) versus 10^4 pfu given in drinking water did protect chicks from challenge (Tripathy and Reed, 1997). In another study, water administered FPV was just as effective as cutaneous administered FPV in protecting chicks from challenge and eliciting a humoral immune response (Nagy et al., 1990).

The extent of viral replication is more important to the immunogenicity of a recombinant antigen than the level of antigen expressed in an infected cell (Andrew et al., 1992). Recombinant FPVs (rFPVs) administered via wing-web stick or subcutaneously elicited antibodies against FPV and expressed foreign antigens. However, rFPVs administered intranasally or conjunctivally elicited no immune response neither against FPV nor against any expressed foreign antigen. Intratracheal administration of the rFPV induced an immune response against the foreign antigen but not against FPV antigens (Boyle and Heine, 1994). A rFPV expressing the hemagglutinin of avian influenza given by wing-web stick, protected chickens from challenge with avian influenza. However, intranasal, eyedrop, and drinking water administration of the rFPV, induced

no detectable avian influenza immunity and little or no protection from challenge (Beard et al., 1992). Some authors have speculated that rFPVs are unlikely to be invasive enough to accomplish immunization by any routes other than wing-web sticks (Beard et al., 1992).

The temporal expression of genes does not affect humoral immunity (Andrew et al., 1992). However, late expressed antigens do not associate with class I major histocompatibility complex (MHC) important for cytotoxic T-lymphocyte recognition. This may be because poxviruses inhibit host protein synthesis and a protein required for processing antigen or producing a functional MHC/peptide complex may be absent late in virus infection. Alternatively, vaccinia encoded protease inhibitors may block MHC/peptide association late in infection (Andrew et al., 1992).

D. Fowlpox virus pathogenesis

Avian poxviruses can be transmitted to susceptible birds by applying a suspension of poxvirus lesion material from infected birds to a scarified comb or denuded feather follicles of the thigh or by the wing-web stick method. Following vaccination with FPV, a "take" can be observed at the site of vaccination. A "take" consists of swelling of the skin or a scab at the

site where the poxvirus was applied and is evidence of successful vaccination (Tripathy and Reed, 1997). "Takes" were first observed in turkeys inoculated intradermally with FPV six days pi and were fully developed ten days pi (Pilchard et al., 1962). Immunity will normally develop in 10-14 days pi. Antibody titers reach a peak 4 weeks pi (Nagy et al., 1990). In turkeys given multiple inoculations of FPV, neutralizing antibodies were developed two weeks after the initial inoculation and continued irregularly for seven weeks or more (Pilchard et al., 1962).

Grossly, local epithelial hyperplasia involving the epidermis and feather follicle are evident. Primary lesions appear by day 4 pi. Papules are formed by day 5 or 6 pi followed by a vesicular stage with the formation of thick lesions. Adjoining lesions may coalesce and become rough gray or dark brown. After about two weeks, lesions are inflammed and hemorrhagic at their bases. Formation of a scab over the lesion surface may last another 1-2 weeks (Tripathy and Reed, 1997). Histologically, there is hyperplasia of the epithelium and enlargement of cells with associated inflammatory changes. Characteristic eosinophilic A-type cytoplasmic inclusion bodies (Bollinger bodies) are readily observable in infected cells (Tripathy and Reed, 1997).

LEGENDS

FIGURE 1. Transcription pattern for adenoviral gene expression. The linear dsDNA adenovirus genome is represented by a gray shaded rectangle. The genome is marked with map units. E1a is the first gene transcribed and translated. The E1a protein *trans* activates other early transcription units. The major late promoter (MLP) is active early but only for the transcription of L1. Viral DNA replication proteins are produced from the E2 transcription unit. Dashed lines indicate the distance from the promoter to the message body. After DNA replication, late gene transcription begins. Most are transcribed in a single run-on transcript driven by the MLP. pIX and pIVa2 lie outside the major late transcription unit (MLTU) and have individual promoters which drive transcription. The MLTU is differentially spliced and polyadenylated to yield most of the viral late mRNAs. The adenovirus tripartite leader sequences are spliced onto the 5' end of MLTU-derived mRNAs. Adapted from Bridge and Pettersson, 1996.

TABLE 1. Antigenic determinants associated with the major adenovirus structural proteins. The protein name and numerical designation are given along with the antigenic determinants associated with the protein. The specificity of the antigen is given along with the location of the antigen in the virion, if known. Adapted from Philipson, 1983.

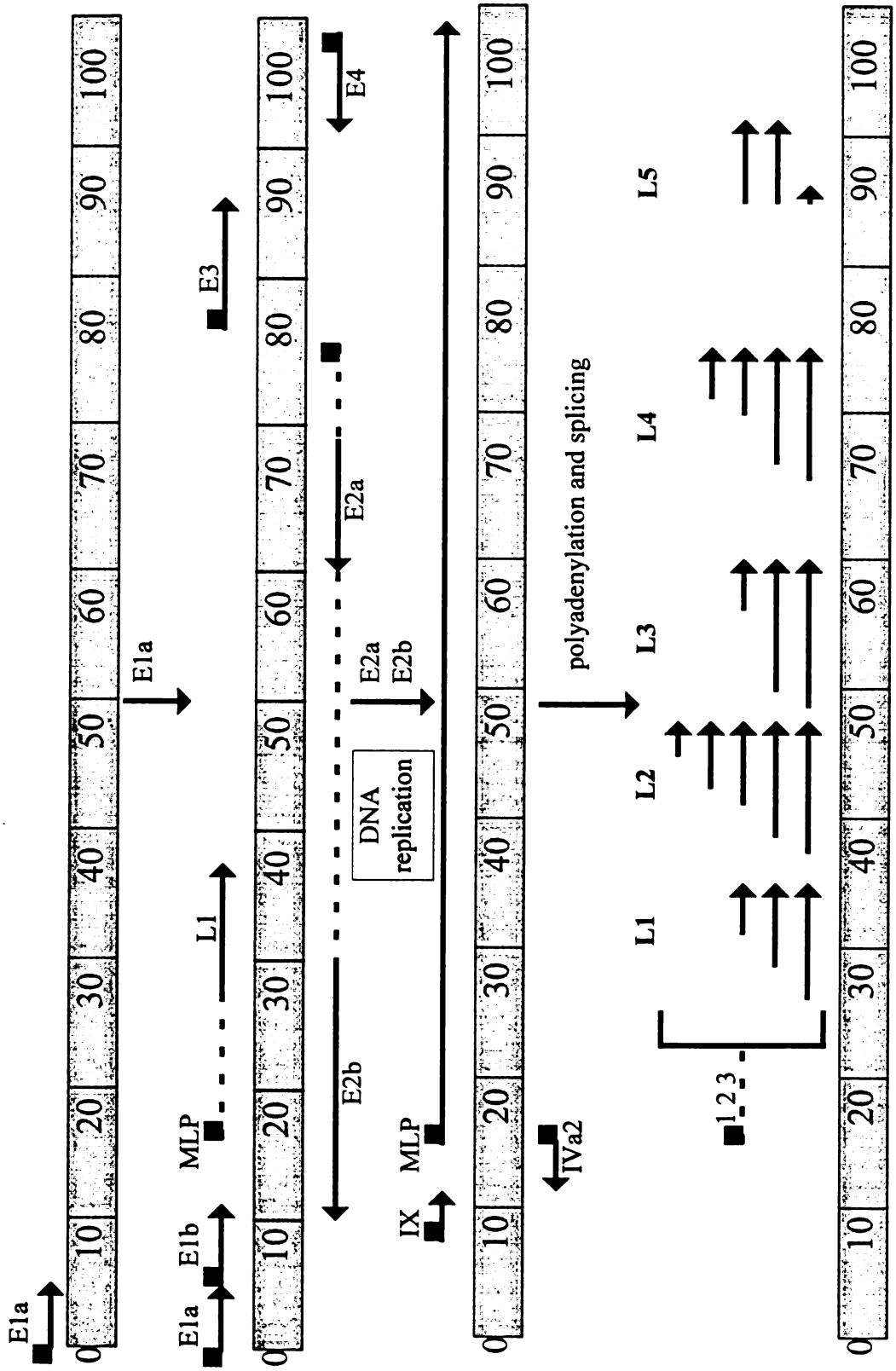

Figure 1. Adenovirus transcription.

Table 1. Antigenic determinants associated with the adenovirus capsid proteins.

Protein		Antigens		
	polypeptide	designation	specificity	remarks
Hexon	II	α	genus	Oriented towards the inside of the virion
		–	inter- and intrasubgenus	
		ε	species	On the surface of the virion
Penton base	III	β	genus, inter- and intrasubgenus	Carries toxin acitivity
Fiber	IV	γ	species	Reacts with hemagglutinin inhibition antibody (associated with knob region)
		–	intersubgenus	
		δ	intrasubgenus	Located at the proximal part of the fiber; only present in adenoviruses with long fibers
Major core protein	VII	–	genus and species	
Other virion polypeptides	IIIa	–	genus	
	IX	–	genus and species	

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Chapter 2

PHYLOGENETIC COMPARISONS OF AVIADENOVIRUSES

Abstract:

The aviadenoviruses are divided into three serogroups: types I, II, and III. Hexon, 100 kD folding protein, and penton sequences from all three serogroups of aviadenoviruses were compared to each other and to selected mastadenoviruses. This analysis shows that the aviadenoviruses are only distantly related. Type II and type III aviadenoviruses are more closely linked to each other than to the prototype virus, CELO virus. In addition, though the relationships between the aviadenoviruses is distant, they are more closely linked to each other than to mastadenoviruses with the exception of ovine adenovirus type 287, as previously reported (Harrach et al., 1997).

Introduction:

Adenoviruses are non-enveloped, icosahedral viruses, 70-90 nm in diameter with a linear double stranded DNA viral genome (Wigand et al.,

1982). The Adenoviridae is divided into two genera: mastadenoviruses and aviadenoviruses (Wigand et al., 1982). The mastadenoviruses have a mammalian host range and the aviadenoviruses infect avian species. The aviadenoviruses are further subdivided into three serogroups: type I, II, and III. Chick embryo lethal orphan (CELO) virus (fowl adenovirus 1{FAV 1}), other FAVs, and quail bronchitis virus are all type I aviadenoviruses. Hemorrhagic enteritis virus (HEV), marble spleen disease virus (MSDV), and avian adenosplenomegaly virus (AASV) are the three type II aviadenoviruses. Egg drop syndrome 76 virus (EDSV) is the only known type III aviadenovirus (Monreal, 1992, McFerran et al., 1997). The aviadenoviruses are divided on the basis of group-specific antigen reactions. Group I or type I aviadenoviruses share a common group antigen. Group II or type II aviadenoviruses share a group antigen distinct from the group antigen of type I aviadenoviruses. Group III or type III aviadenoviruses partially share the type I group antigen (McFerran et al., 1997).

The type I aviadenoviruses, including the prototype aviadenovirus, CELO virus, have larger genomes than mastadenoviruses (Sussenbach, 1984). CELO virus has a genome of 43.8 kilobases (kb) (Chiocca, et al., 1996), slightly larger than mastadenoviral genomes which range from

approximately 30 kb to 36 kb. Another type I aviadenovirus, FAV 8, has a genome size estimated to be 44.7 kb (Clavijo et al., 1996). In contrast to the type I aviadenoviruses, the type II and type III aviadenovirus genomes fall within the mastadenovirus size range. The genomes of type II aviadenoviruses, including HEV, are approximately 25 kb in length (McQuiston et al., 1995, McFerran et al., 1997, Jucker et al., 1996). The type III aviadenovirus, EDSV, has a genome length of 33.4 kb (Brandt et al., 1997).

The organization of the adenoviral genome is highly conserved among mastadenoviruses (Sussenbach, 1984). The recently published CELO genome sequence shows that its genomic organization has several differences from the typical mastadenovirus organization (Cai and Weber, 1993, Chiocca et al., 1996). The central portion of the genome, where the structural protein genes are located, is conserved between CELO virus and the mastadenviruses. The genes for the hexon, penton base, pIIIa, fiber, pVI, pVII, pVIII, and the E2 region are present and in the same locations in the CELO virus genome as in mastadenoviral genomes (Chiocca et al., 1996). There is, however, 5 kb of sequence at the left end and 15 kb at the right end of the CELO virus genome with little or no sequence identity with

mammalian adenoviruses. In addition, there are no E1, E3, and E4 regions identified in CELO virus. However, there are several open reading frames unique to CELO virus which are recognized at the left and right ends of the genome. One of these open reading frames (ORFs), ORF 8 or GAM-1, has been determined to share an anti-apoptotic function with the E1b 19k protein and Bcl-2 (Chiocca et al., 1997). GAM-1 is located in the 15 kb of sequence unique to CELO at the right end of the genome. The virus associated (VA) RNA is found at the right end of the CELO virus genome (Larsson et al., 1986, Chiocca et al., 1996) and a dUTPase at the left end, opposite to mastadenoviruses (Chiocca et al., 1996). These changes have led to speculation that the CELO virus has undergone some rearrangement of the genome around the central block of structural genes in which the immortalizing and transforming genes of the E1 region have been moved to the left end of the genome and other genes to the right end of the genome (Chiocca et al., 1996). GAM-1 bears no DNA or amino acid sequence similarity to the E1 region which carries the genes involved in immortalization and transformation in other adenoviruses.

In contrast to CELO virus, EDSV has most of the same transcription units described in mastadenoviruses in the same locations, although the E3

transcription unit has not been located and there are several ORFs at the right end of the genome to which no function has been assigned (Brandt et al., 1997). In the information available on the genomic organization of HEV, the E1b region, penton base, pVI, and core protein genes are all in the same locations as they are in mastadenoviruses (McQuiston, et al., 1995). Although the information is sparse, the presence of an E1b transcription unit near the left end of the genome, suggests that HEV has not undergone the same rearrangement of the genome seen in CELO virus. Although some authors have speculated that HEV has undergone significant genomic rearrangements in comparison to mastadenviruses (Jucker et al., 1996).

The differences between aviadenoviruses including genome size, and organization, imply a distant phylogenetic relationship. In a comparison of the 23 kD protease gene from the L3 transcription unit, EDSV was found to cluster with ovine adenovirus 287 (OAV) and bovine adenovirus type 7 (BAV 7) but did not cluster with CELO virus (Harrach et al., 1997). Phylogenetic comparisons between other aviadenoviruses have been limited by the lack of sequence data available for the type II aviadenoviruses. For the first time, sequence data on the type I, type II, and type III aviadenoviruses is available for analysis. In this report, sequences from all

three of the avian adenovirus serotypes are compared to each other and to published mastadenovirus sequences.

Materials and methods:

DNA preparation. HEV virus was grown in the RP19 cell line as described by Nazerian and Fadly (Nazerian and Fadly, 1982). Briefly, RP19 cells less than 20 passages, were grown for two passages in 65% Leibovitz-McCoy medium, 20% chicken serum (Gibco BRL, Life Technologies, Grand Island, NY; lot #35N1850), 10% bovine fetal serum, 5% tryptose phosphate broth, penicillin, streptomycin, and amphotericin B. For remaining passages, cells were grown in 82.5% Leibovitz-McCoy medium, 10% chicken serum (Gibco BRL, Life Technologies, Grand Island, NY; lot #35N1850), 5% bovine fetal serum, 2.5% tryptose phosphate broth, penicillin, streptomycin, and amphotericin B. Infected cells were harvested, sonicated four times with a Braun-sonic 2000 U sonicator (Bob Braun Biotech, Inc., Allentown, PA) for 20 seconds and incubated with DNase and RNase A in the presence of 10 mM $MgCl_2$ for 3-6 hours at 37 C to digest cellular DNA and RNA. The viral capsid was lysed by incubation with SDS and Proteinase K at 37 C for 3-6 hours. The viral DNA was extracted with

phenol/chloroform extraction and precipitated with 100% ethanol and NaCl at -20 C. DNA samples were washed with TE to remove any residual salts using a Centricon 30 concentrator (Amicon Inc., Beverly, MA). Single digests of purified DNA were done with BamHI, EcoRI, BglII, HindIII, and PstI restriction enzymes.

Southern blotting. Digested DNA was transferred to a negatively charged nylon membrane using Southern blotting technique (Ausubel et al., 1993). Briefly, the agarose gel was placed on top of a stack consisting of the pre-wetted nylon membrane, a pre-wetted Whatman blotting paper, 2 pieces of dry blotting paper, and a stack of dry paper towels. Two pre-wetted wicks were placed one end in a well of 10X SSC and the other end atop the gel. The transfer was run overnight. The transferred total DNA was covalently bound to the membrane by baking at 80 C for 2 hours. Blots were probed with digoxigenin labeled DNA probes using the Genius kit from Boehringer Mannheim (Indianapolis, IN). Hybridization was performed following the Genius protocol.

A probe for the hexon gene was generated with mixed PCR primers designed from regions of sequence homology from published mastadenovirus sequences (primer 1: GGG GGA TCC ATG TGG AAY

CAR GCN RT; primer 2: GGG GAA TTC GGR TTN ACR TTR TCC AT). PCR was performed under standard conditions. Briefly, 1 mM each dNTPs, 1 picoM each primer, 1 ng DNA template, Taq polymerase, 1X PCR buffer and 0.025 mM $MgCl_2$ in a total volume of 100µl were combined for the reaction. Template DNA was denatured for 2 min. at 96 C then cycled 35 times in a MiniCycler (M.J. Research,) with the following procedure: Denaturation, 20 sec., 96 C; Reannealing, 30 sec., 50 C; Extension, 60 sec., 72 C. A final extension stage of 5 min. at 72 C was performed.

Cloning. Fragments of HEV DNA identified to contain the hexon and 100 kD folding protein genes were cloned into linearized pUC18 vectors with compatible cohesive ends. Vectors with identical ends were dephosphorylated with calf intestinal alkaline phosphatase (CIAP) for 30 min. at 37 C. The CIAP was inactivated by heating to 56 C for 15 min. Then the dephosphorylated vector was extracted with phenol/chloroform, ethanol precipitated, and resuspended in TE. The HEV DNA fragments and the vectors were combined in a vector:HEV fragment ratio of 1:10 and ligations were performed overnight at 14 C with T4 DNA ligase and 10X ligation buffer.

Transformation competent TG-1strain *E. coli* were transformed via electroporation (Cell-Porator, BRL, Grand Island, NY) at 400 volts, 4 kila-ohms and a capacitance of 330 microfarads with the ligation mixture and plated on 2YT agar with halogenated indolyl β-D galactoside (Bluogal, Life Technologies, Gibco-BRL, Grand Island NY), isopropyl β-D-thiogalactopyranoside (IPTG; Sigma Chemical Co., St. Louis MO), and ampicillin. Plates were incubated from 16-20 hours at 37 C. Blue, ampicillin resistant colonies were selected and grown in 1.5 ml 2YT medium containing ampicillin for 4-24 hours. Colonies were screened for inserts with minipreps (Ausubel et al., 1993). Positive clones were amplified and DNA extracted and purified with a Qiagen-tip 500 (Qiagen, Chatsworth, CA). DNA was stained with Hoechst dye and quantitated with a DNA fluorometer (Hoefer Scientific Instruments, San Francisco, CA).

Sequencing. Cloned fragments of HEV DNA were sequenced using an automated sequencer (373A DNA Sequencer, Applied Biosystems, Foster City, CA) and dideoxy sequencing methods (Prism, Applied Biosystems, Foster City, CA).

Computer analysis. Hexon, 100 kD folding protein, and penton base nucleotide and amino acid sequences from adenoviruses were taken from

GenBank accessions. The adenoviruses compared and their GenBank accession numbers are listed in Table 2. Alignments and pairwise comparisons of amino acid and DNA sequences were performed using the Pileup program in the GCG package (Version 8.1). Phylogenetic relatedness calculations were done by protein distance calculation based on the Dayhoff PAM matrix, bootstrapping and phylogenies were estimated using the Fitch-Margoliash criteria. A consensus tree for each protein analyzed was calculated and drawn. All analyses were done with the Phylogeny Inference package, version 3.5c by Joseph Felsenstein (1993).

Results and Discussion:

The hexon was identified in the HEV genome by the methods described in fragments: PstI-2, HindIII-1 and HindIII-4, BglII-2, EcoRI-2, and BamHI-2 (Figure 2). The 100 kD folding protein gene was identified using primer walking technique in the HEV genome in fragments PstI-4 and PstI-7, HindIII-1, HindIII-2, and HindIII-8, BglII-1, EcoRI-1, and BamHI-3 and BamHI-4 (not shown). The sequences of the full open reading frames of the HEV hexon and 100 kD folding protein genes are shown in Figures 3 and 4.

Figures 5-7 show the unrooted phylogenetic trees obtained by analysis of the hexon, 100 kD folding protein, and penton base proteins, respectively. They show a clustering of the human adenoviruses which agrees with previously published results (Bailey and Mautner, 1994). Adenoviruses from non-human mammals cluster near the human adenoviruses with the exception of MAV 1 which does not cluster with any of the sequences analyzed. The aviadenoviruses do not form a distinct cluster but do lie farthest from the human adenoviruses. EDSV clusters with OAV, as previously reported (Harrach et al., 1997). Bootstrapping numbers are given at each node and indicate that the consensus tree is statistically accurate for the analysis of the aviadenoviruses. The phylogenetic comparisons presented, agree with previously published comparisons (Bailey and Mautner, 1994, Harrach et al., 1997).

Other authors have hypothesized that HEV is more closely related to Ad2, the mastadenovirus prototype, than to CELO virus, the aviadenovirus prototype (Jucker et al., 1996). The analyses presented here do not support such a claim. The phylogeny of hexon, penton, and the 100kD folding protein all show the aviadenoviruses clearly share more homology with each other than the mastadenoviruses with the exception of OAV. From

this analysis, it seems likely that OAV represents an aviadenovirus-like mastadenovirus rather than EDSV representing a mastadenovirus-like aviadenovirus. BAV 7, not analyzed here, may also fall into the aviadenovirus-like mastadenoviruses based on previously published work (Harrach et al., 1997).

Hemorrhagic enteritis of turkeys was first described in 1937 by Pomeroy and Fenstermacher (Pomeroy and Fenstermacher, 1937). This first outbreak occurred consisted of 35 turkeys, 7-12 weeks old from widely separated and variously sized flocks in Minnesota. Gale and Wyne reported the next two outbreaks of HE in 1957 in two flocks of confinement raised turkeys in Ohio although they report HE had recurred sporadically in the intervening 20 years (Gale and Wyne, 1957). Hemorrhagic enteritis emerged as a severe problem in the turkey industry and reached epidemic proportions in Texas in the early 1960s and in Virginia in the mid-1960s (Gross and Moore, 1967, McFerran et al., 1997).

The sudden appearance of HEV in Minnesota remains unexplained. A reservoir host for type II aviadenoviruses has not been identified. Type II aviadenovirus antibodies have not been detected in surveys of wild birds including wild turkeys (Domermuth et al., 1977, Hopkins et al., 1990). This

phylogenetic comparison suggests that HEV has diverged significantly from the other aviadenoviruses and from mastadenoviruses. And it seems unlikely that HEV arose from a type I or a type III aviadenovirus in 1937. There are two logical sources of type II aviadenoviruses. One explanation is that they existed and still exist in an unidentified population of wild birds. This explanation seems implausible since more than 40 species of wild birds have been surveyed (Domermuth et al., 1977, Hopkins et al., 1990) with no type II aviadenovirus antibodies detected. Second, it is possible that HEV existed in domestic turkeys prior to 1937 but did not become a recognizable problem until turkeys were raised intensively. The original outbreak, however, did not occur in intensively raised turkeys. However, the disease did not reach epidemic proportions until the 1960's when turkeys were being raised more intensively. The evolutionary origin of type II aviadenoviruses remains a mystery.

LEGENDS

TABLE 2. GenBank accessions used for phylogenetic comparisions.

FIGURE 2. Southern blot of HEV genomic DNA probed with a PCR generated fragment of the HEV hexon gene. DNA marker sizes are given at the left. Lane 1 is PstI digested HEV DNA. Lane 2 is HindIII digested HEV DNA. Lane 3 is BglII digested HEV DNA. Lane 4 is EcoRI digested HEV DNA. Lane 5 is BamHI digested HEV DNA.

FIGURE 3. Nucleotide sequence of the hexon gene of HEV.

FIGURE 4. Nucleotide sequence of the 100 kD folding protein gene of HEV.

FIGURE 5. Phylogeny of adenoviruses, hexon. Numbers at the branch points indicate bootstrapping results. Avian and human adenoviruses are indicated.

FIGURE 6. Phylogeny of adenoviruses, 100 kD folding protein. Numbers at the branch points indicate bootstrapping results. Avian and human adenoviruses are indicated.

FIGURE 7. Phylogeny of adenoviruses, penton base. Numbers at the branch points indicate bootstrapping results. Avian and human adenoviruses are indicated.

Table 2. GenBank accessions used for phylogenetic comparisons.

Adenovirus	Host species	hexon	100 kD	penton
Ad2	human	J01917	J01917	J01917
Ad4	human	X84646		
Ad7	human	X76551		
Ad12	human	X73487	X73487	
Ad40	human	L19443	L19443	L19443
Ad41	human	X51783	M19540	
EAV 1	equine	M86664		
CAV 1	canine	U55001	U55001	U55001
CAV 2	canine	U77082		U77082
BAV 3	bovine	K01264		
OAV	ovine	U40837	U40837	U40837
PAV 3	porcine	U34592		U24432
MAV 1	murine	M81889	U23770	U95843
CELO	avian: chicken	U46933	U46933	U46933
FAV 10	avian: chicken	L07890		
HEV	avian: turkey			U28139
HEV (A)	avian: turkey			U31805
EDSV	avian: duck	Y09598	Y09598	Y09598

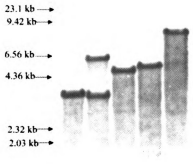

Figure 2. Southern blot of HEV genomic DNA probed with a PCR generated fragment of the HEV hexon gene.

Figure 3. Nucleotide sequence of the hexon gene of HEV.

ATGGACATATCAAATGCTACGCCAAAACTTGATATATTCCACATAGCTGGA
CCAGATGCTTCAGAATATCTTTCAGAAAATCTCGTTAATTTCATCTCCAGT
ACAGAATCGTATTTTCCAATTAATAAAAAATTTAGAGAAACAATTGTAGCA
CCAACAAAAGGTGTGACGACAGAACAATCTCAGAAATTGCAAGTTAAAATT
GTTCCAACTTTGACACAAGATTTAGAAAATAGTTTTACTGCTAGATTTACT
ATTGCTGTTGGCGATGGTCGGGTTTTGGATATGGGAAGTACGTATTTTGAT
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GCATATAATCCTCTAGCTCCAAGGTCAGCTCAATTTAATAATATTAAAACT
GTGGGTGGTAAAACATATTTGACTGCTCAAGCTACTAAATTTTTTTCAACA
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TTAGTTCCTTCACCTAATACTGGTTCAGCAGAAAGTTCTTTTGATCCTACA
ACAGAGGGAGCTAGTTGTAGAGCTATAACACTAGGCAGTTCTGTAACAGAT
GCAACTTGTTATGGAGCTTATACACCTATTCAAAATGCTAATGGTTCAATT
TTACCTCCATCTGTTACGCCTGATAAAAAATTTGCCGATGCTGGTAAATCT
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TATCCAGATACTAGAATAGTTGCTTATGACTCTACTGATAAAATAGCAACT
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TTGATGTATTATGATAATGGTGCACATAGTGGTTCTTTGGCTACAGAAACA
GGAGATATAAATTTGGTAGAACAATTGCAAGATAGAAATACAGAAATTAGT
TATCAATATATGTTAGCGGATTTGATGAGTAGGAATCATTATTATAGTCAG
TGGAATCAACCTGTAGATGATTATGATTTAAATGTTAGAGTACTTACAAAT
ATTGGTTATGAAGAGGGTCCTCCAGGTTACTGTTATCCAAGCACAGGCATG
GGCAACTATCCTAATACTGTCATGTCGGTTGGGACATTAGTGGATAATAAT
GGTACAACTGCTACAACAACGTCAAATACTGTAGCTGTGATGGGTTTTGGC
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CAAGGTACTAGTGAAACTGATCCAACAACTTATAGTTATATGAATAGTAGG
CTTCCTAATGTGAATATGGCTGATCTCTTTACACATATTGGCGGGCGTTAT
AGTTTGGATGTAATGGATAATGTTAATCCTTTTAATCATCATAGAAATAGA
GGTTTGCAATATAGAAGTCAAATTTTGGGTAATGGTAGAAATGTCCGTTTT
CATATTCAGGTACCTCAGAAATTTTTTGCTATTAAGAATCTATTGTTACTT
CCTGGAACTTATAGTTATGAATGGTGGTTCAGGAAAGATCCAAACTTAGTG
CTACAGTCTACGTTGGGAAATGATTTAAGAAAAGATGGAGCAAGCATTCAG
TTAGCAGTTATTAGTCTTTATGCGAGTTTTTTTCCTATGGATCACGCTACT
TGTAGTGAGCTTATTTTAATGCTTAGAAACGATCAAAATGATCAAACTTTT
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TTTTATCTTTCTCACACTTTTAACTCTATGTCTATTTTGTTTGATTCAGCA
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GGAACTGCTTCAGTA

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CAAGTTTGAATCAGATATGTTTGTGATTTCAGACTGTCTTGGGTCCGAAG
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TTTTAATTGAAAAAGGTTCTGGTATTTATTTGGATCCCCAAACGGGAGAT
AACTTAAGTGATGCTAAATTGTTTCACAGCCCAGAAGAGGCAGCAGTGGC
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Figure 4. Nucleotide sequence of the 100 kD folding protein gene of HEV.

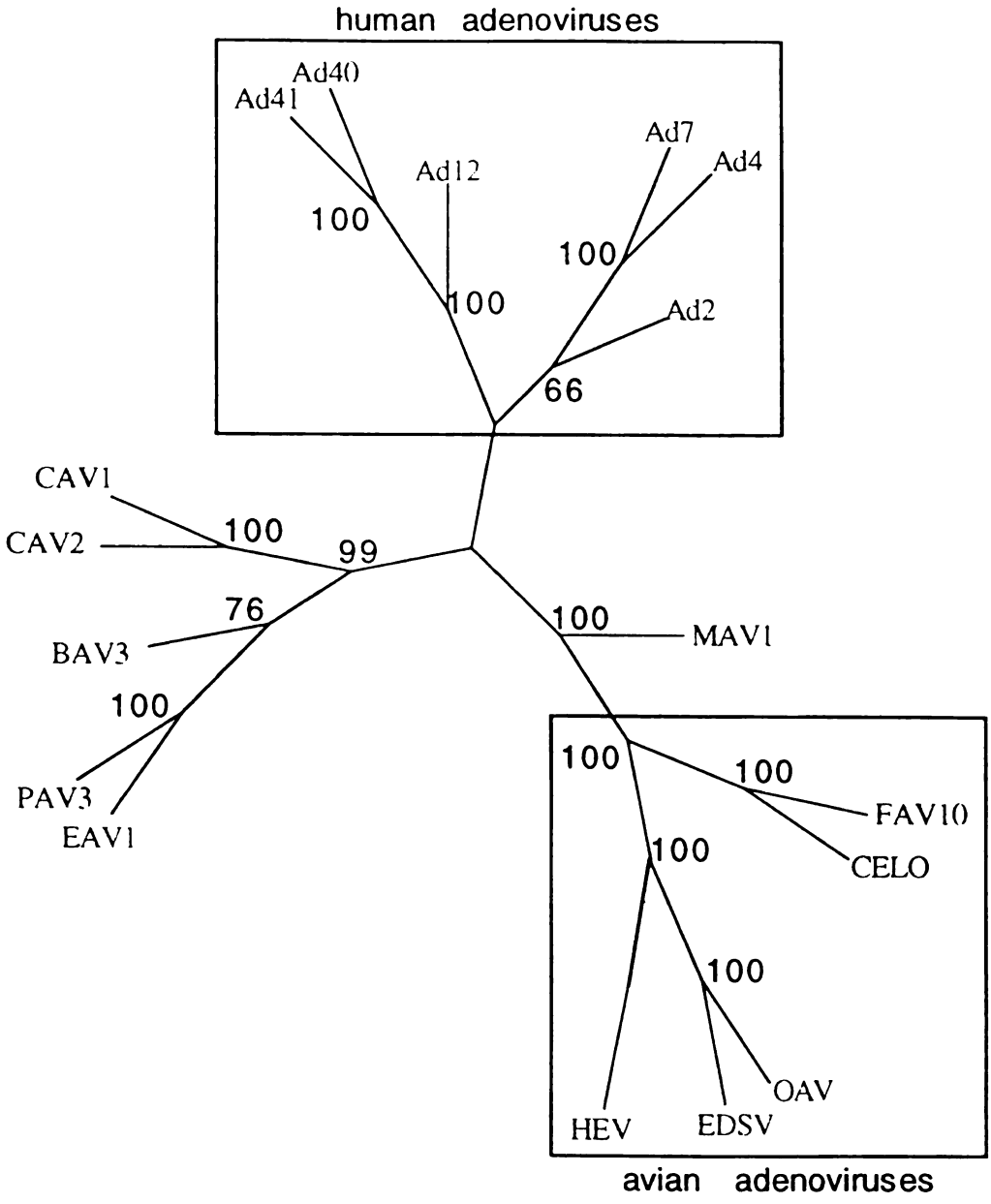

Figure 5. Phylogeny of hexon proteins.

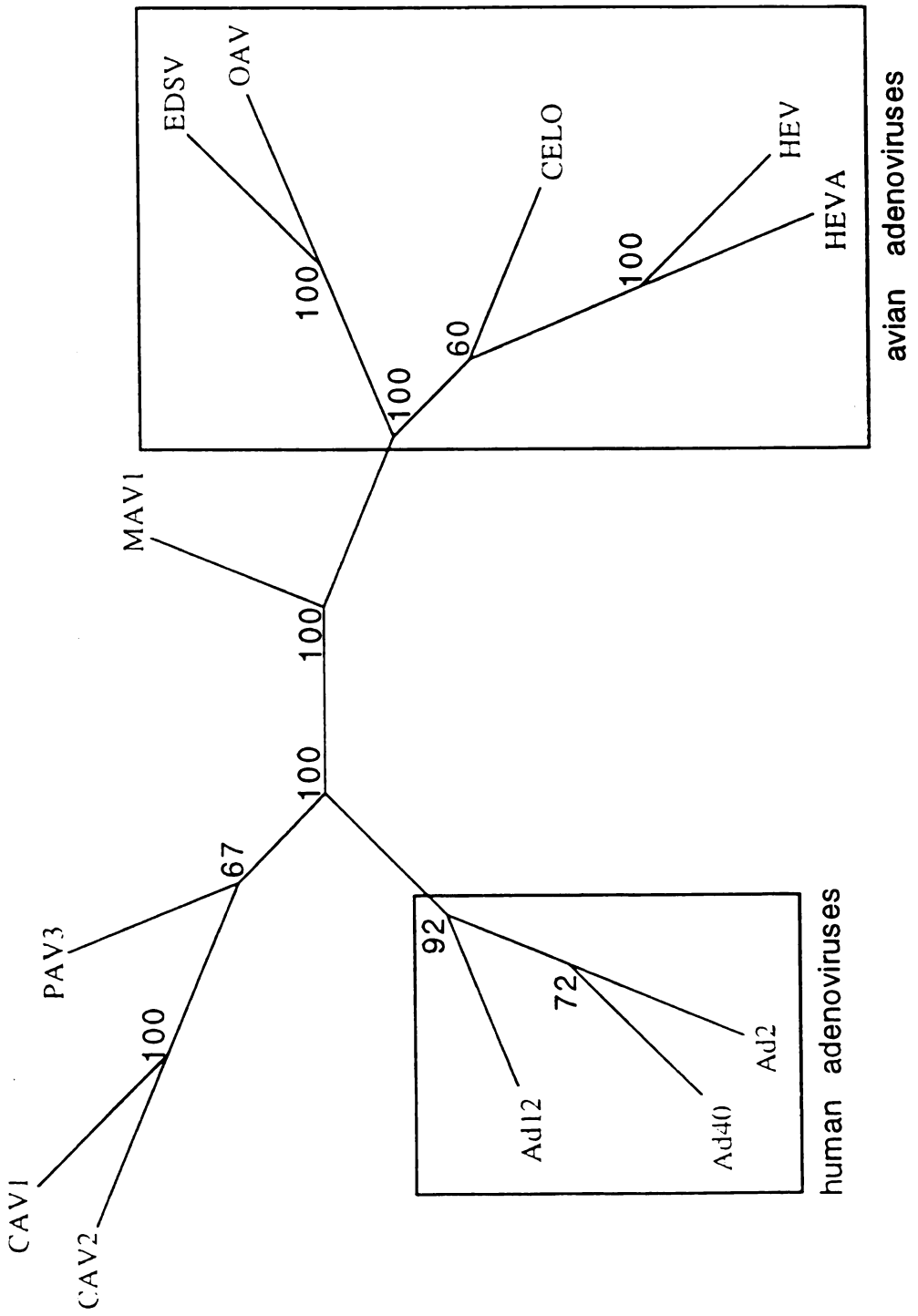

Figure 6. Phylogeny of 100 kD folding proteins.

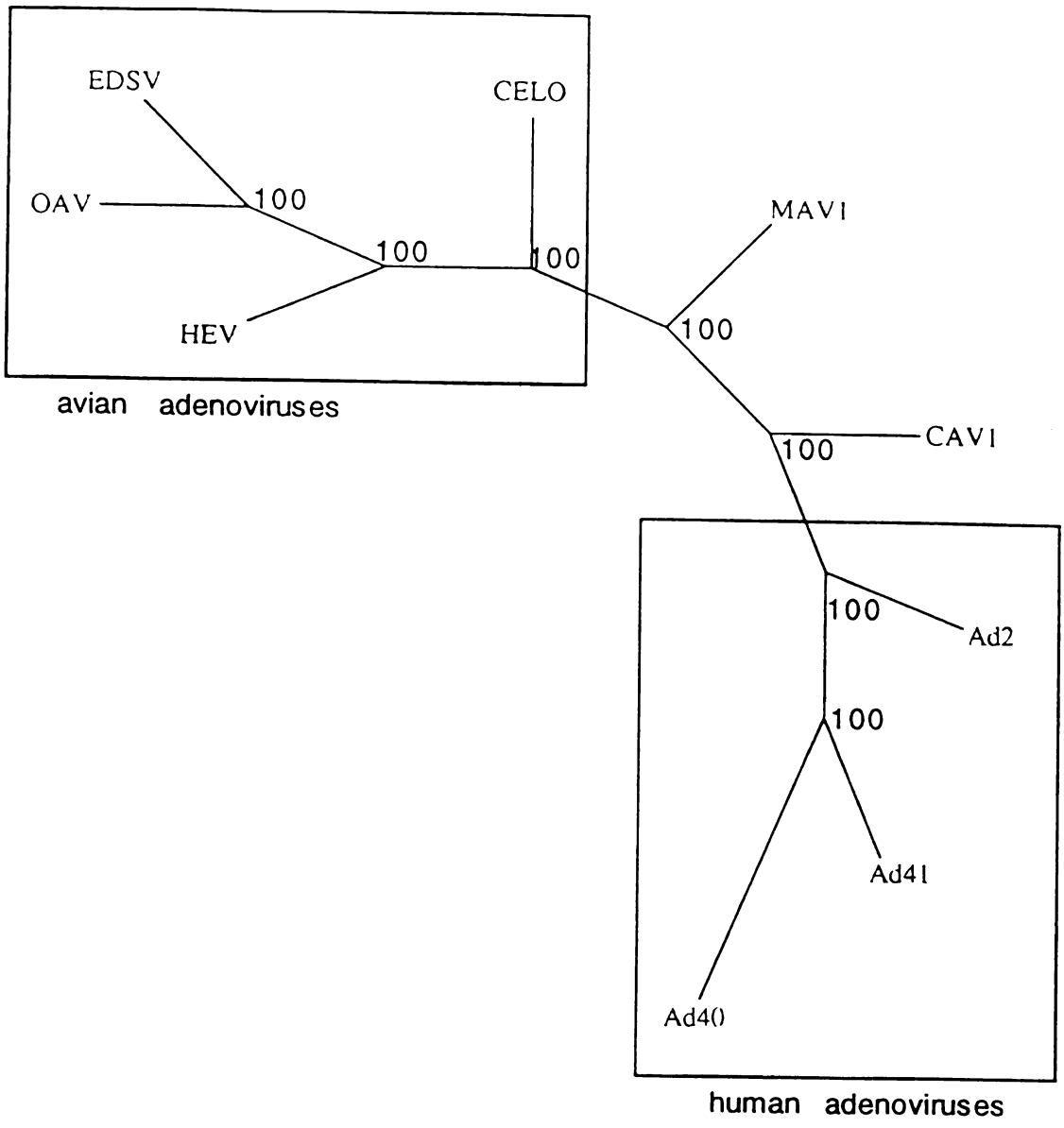

Figure 7. Phylogeny of penton base proteins.

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Chapter 3

CHARACTERIZATION OF A RECOMBINANT FOWLPOX VIRUS EXPRESSING THE NATIVE HEXON OF HEMORRHAGIC ENTERITIS VIRUS

Abstract:

The structure of the icosahedral adenovirus capsid is highly conserved among Adenoviridae. In its native form, the hexon is the major capsid protein. The nascent hexon requires the 100 kD folding protein to fold into its native, trimeric form but may also require other adenoviral proteins. In this work, the hexon and 100 kD folding proteins were co-expressed in a fowlpox virus (FPV) vector. In the recombinant FPVs (rFPVs) in which the hexon and 100 kD folding protein genes are cloned head to tail, the native hexon could be detected with indirect immunofluorescence and immunoprecipitation. The rFPVs expressing both the hexon and 100 kD folding protein were tested in chickens for their ability to elicit a humoral immune response. The FPV-@X100 construct in

which the 100 kD folding protein gene follows the hexon gene in a head to tail fashion, elicited the largest response. The HEV commercial vaccine elicited higher and longer lasting anti-HEV titers than FPV-@X100. Humoral immunity was also compared in turkeys inoculated with rFPVs expressing the hexon alone, the 100 kD folding protein alone, or expressing both genes in different configurations. No anti-HEV humoral immune response was detected in turkeys inoculated with the rFPVs expressing the hexon alone or the 100 kD folding protein alone. The anti-HEV humoral immune response in turkeys inoculated with FPV-@X100 was compared to the humoral response of turkeys given a commercial HEV vaccine.

Introduction

Hemorrhagic enteritis of turkeys was first described in 1937 by Pomeroy and Fenstermacher (Pomeroy and Fenstermacher, 1937). The disease is characterized by hemorrhagic enteritis most severe in the duodenum (Pomeroy and Festermacher, 1937, Domermuth and Gross, 1991, McFerran et al., 1997) as well as a grossly enlarged, mottled spleen (Domermuth and Gross, 1991, McFerran et al., 1997). Hemorrhagic enteritis is caused by a type II aviadenovirus, hemorrhagic enteritis virus

(HEV) (Domermuth and Gross, 1991, Monreal, 1992, McFerran et al., 1997). HEV of turkeys is closely related to the other type II aviadenoviruses, marble spleen disease virus (MSDV) of pheasants and avian adenovirus splenomegaly virus (AASV) of chickens (Domermuth and Gross, 1991, Monreal, 1992, McFerran et al., 1997). They differ in pathogenicity for their target species, but not in their ability to infect the different species of poultry (Domermuth and Gross, 1991, Monreal, 1992, McFerran et al., 1997).

The capsid of adenoviruses is icosahedral and is composed of 252 capsomeres, 240 of which are hexons and 12 of which are pentons (Philipson et al., 1975). There are 180 hexons which make up the 20 triangular faces of the icosahedron and 60 total hexons which surround the pentons at the twelve vertices. The hexon found in the adenoviral capsid is a trimer (Grütter and Franklin, 1974, van Oostrum and Burnett, 1985) of stably but non-covalently associated hexon polypeptides (Cepko and Sharp, 1983, Cornick et al., 1973). The trimeric, native hexon is recognized by different antibodies than is the nascent hexon (Cepko et al., 1981, Fortsas et al., 1994). Hexons represent the dominant viral protein both in the virion

and in the infected cell and are, therefore, the major antigenic component (Monreal, 1992, Philipson et al., 1975).

Early descriptions of the native hexon were of a solid sphere (Horne et al., 1959, Valentine and Pereira, 1965). Later the hexon was described as a hollow sphere or polygon (Wilcox and Ginsberg, 1963, Petterson et al, 1967). More recent reports show the hexon has a threefold symmetry based on electron microscopy and crystal structure. The hexon consists of two structural parts including a triangular top 64 angstroms tall with three towers and a pseudo-hexagonal base 52 angstroms tall with a central cavity (Athapilly et al., 1994, Roberts et al., 1986). The lowest 1 nm of the hexon facing the DNA core, is 7.5 nm in diameter with an axial hole 3.5 nm in diameter. The mid 1-5.2 nm is hexagonal with an 8.9 nm side while the top 5.2-11.6 nm is triangular with a 7.5 nm side (Philipson, 1983). The internal surface of the hexon is hydrophobic while the external surface is negatively charged (Philipson, 1983). From the pseudo-hexagonal symmetry of the base arises two kinds of vertical hexon to hexon contact faces which alternate around the base. These are the A face, under each tower, and the B face, lying between the towers (Philipson et al., 1975, Roberts et al., 1986). Each contact is A face to B face between hexon subunits in the viral capsid

(Roberts et al., 1986). The three identical hexon polypeptides are tightly interwoven at the interfaces. Each tower is formed from three loops, one from each hexon polypeptide. (Roberts et al., 1986, Athapilly et al., 1994)

The nascent hexon requires the co-expression of the 100 kD folding protein to achieve the complex configuration of the hexon. The 100 kD folding protein plays roles in the formation of hexon trimers (Morin and Boulanger, 1986) and in the transport of the hexon trimers to the nucleus (Gambke and Deppert, 1983, Cepko and Sharp, 1983, Oosterom-Dragon and Ginsberg, 1981, Williams and Ustacelebi, 1971). The 100 kD folding protein also has a role as a translational activator (Adam and Dreyfuss, 1987, Hayes, et al., 1990, Riley and Flint, 1993) which is unrelated to its role as a hexon folding protein. Much of the work on the nature of the 100 kD folding protein and hexon polypeptide interaction has been done using temperature sensitive (ts) adenovirus mutants (Grodzicker et al., 1977, Cepko and Sharp, 1982, Cepko and Sharp, 1983, Young et al., 1984). Two types of ts mutants in Ad5 have been defined: "hexon minus" mutants (Russell et al., 1972, Leibowitz and Horwitz, 1975) which fail to produce hexons at non-permissive temperatures and "transport" mutants (Russell et al., 1972, Kauffman and Ginsberg, 1976) which produce hexon trimers

which are not transported to the nucleus. The hexon minus mutants have mutations in the hexon gene while the transport mutants have mutations which map to the L4 transcription unit, specifically to the 100 kD folding protein gene (Williams and Ustacelebi, 1971, Williams et al., 1974).

Based on the literature, it seems clear that expression of the native hexon requires co-expression of the hexon and 100 kD folding protein gene (Cepko and Sharp, 1983, Oosterom-Dragon and Ginsberg. 1981, Williams and Ustacelebi, 1971). However, it is not clear whether or not the hexon and 100 kD folding protein are the only essential adenovirus proteins for native hexon production In this report the native form of the hexon protein is produced by co-expression of the hexon and 100 kD folding protein genes in a fowlpox virus (FPV) vector.

Materials and methods:

DNA preparation. HEV was grown in the RP19 cell line (Nazerian et al., 1982) as described by Nazerian and Fadly (Nazerian and Fadly, 1982). Briefly, RP19 cells less than 20 passages, were grown for 2 passages in 65% Leibovitz-McCoy medium (LM), 20% chicken serum (Gibco BRL, Life Technologies, Grand Island NY), 10% bovine fetal

serum, 5% tryptose phosphate broth, penicillin, streptomycin, and amphotericin B. For remaining passages, cells were grown in 82.5% LM, 10% chicken serum, 5% bovine fetal serum, 2.5% tryptose phosphate broth, penicillin, streptomycin, and amphotericin B. Infected cells were harvested, sonicated four times with a Braun-sonic 2000 U sonicator (Bob Braun Biotech, Inc., Allentown, PA) for 20 seconds and incubated with DNase and RNase A in the presence of 10 mM $MgCl_2$ for 3-6 hours at 37 C to digest cellular DNA and RNA. The viral capsid was lysed by incubation with SDS and Proteinase K at 37 C for 3-6 hours. The viral DNA was extracted with phenol/chloroform extraction and precipitated with 100% ethanol and NaCl at -20 C. DNA samples were washed with Tris-EDTA (TE) to remove any residual salts using a Centricon 30 concentrator (Amicon Inc., Beverly, MA).

Construction of recombinant FPVs. The hexon and 100 kD folding protein genes were cloned from the HEV genome as described (Chapter 2). Recombinant FPVs were constructed using previously published methods (Ogawa et al., 1990, Yanagida et al., 1992). The hexon gene was modified in the following way. A BamHI restriction site was inserted immediately 5' to the start codon of the hexon gene using PCR site-directed mutagenesis.

A 400 bp PCR generated fragment was ligated to a fragment of genomic DNA containing the remainder of the coding region of the hexon gene. The hexon gene was cloned using the unique BamHI and a SalI restriction sites. The 100 kD folding protein gene was modified in the following way. A BglII restriction site was inserted immediately 5' to the start codon of the 100 kD folding protein gene using PCR site-directed mutagenesis. This 150 bp fragment was ligated to a fragment of genomic DNA containing the remainder of the coding region of the 100 kD folding protein gene. The 100 kD folding protein gene was cloned using the unique BglII and SalI restriction sites. Both genes were cloned individually into the transfer vector in which a synthetic late/early fowlpox promoter was cloned in frame with each gene. Procedures for the transfection of plasmids into FPV-infected cells with electroporation, have been described (Ogawa et al., 1990, Nazerian et al., 1992, Yanagida et al., 1992, Calvert et al., 1993, Yoshida et al., 1994). See Figure 8 for diagrams of recombinant FPVs.

Southern blotting. Digested DNA was electrophoretically separated in a 0.8% agarose gel. The DNA was transferred to a negatively charged nylon membrane using Southern blotting technique (Ausubel et al., 1993). Briefly, the agarose gel was placed on top of a stack consisting of the pre-

wetted nylon membrane, a pre-wetted Whatman #2 blotting paper, 2 pieces of dry blotting paper and a stack of dry paper towels. Two pre-wetted wicks were placed one end in a well of 10X SSC (1.5 M NaCl, 0.15 M trisodium citrate) and the other end atop the gel. The transfer was run overnight. Transferred DNA was covalently bound to the membrane by baking at 80 C for 2 hours. Blots were probed with digoxigenin labeled DNA probes using the Genius kit from Boehringer Mannheim, Inc. (Indianapolis, IN).

Western blots. HEV was grown in RP19 cells until cytopathic effect was observed in approximately 10% of the infected cells. Cytoplasmic proteins were extracted in the following way. The cells were harvested, and washed with PBS twice. The cell pellet was resuspended in TEN buffer (10mM Tris-HCl [pH 8.0], 1mM EDTA, 100 mM NaCl), centrifuged at 10,000 x g for 15 seconds, and the supernatant removed and discarded. Cytoplasmic proteins were extracted in the following way. The cell membranes were lysed with 0.1% Nonidet P-40 (Sigma Chemical Co., St.Louis, MO) in TEN buffer, 10-15 minutes on ice. The lysed cells were then centrifuged at 10,000 x g for 30 sec. and the supernatant containing cytoplasmic proteins was collected.

Western blotting was performed according to published methods (Ausubel et al., 1993). Briefly, the cytoplasmic proteins were boiled for 5 min. and centrifuged for 5 min. This extract was loaded and run on a 6% SDS-Page gel and fixed in destain solution (10% glacial acetic acid and 25% methanol in water). The proteins were transferred to Hybond-C extra supported nitrocellulose membrane (Amersham Life Science) in a Bio-Rad Trans-blot cell (Richmond, CA) transfer tank. The transfer was performed at 100 volts and 0.4 amps for 1 hour in Tris glycine buffer (0.25 M Tris, 0.192 M glycine, 20% methanol in water). The membrane was blocked with blocking solution (5% nonfat dry milk in Tris buffered saline [TBS]) for 1 hour at room temperature. The membrane was then incubated with polyclonal turkey or chicken antibody diluted 1:100 in blocking solution. The membrane was washed thrice (15 min. washes) with washing solution (0.01% Tween 20 in TBS [TBS-T]). Goat anti-turkey immunoglobulin G (IgG) coupled to alkaline phosphatase (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was diluted 1:800 in blocking solution and incubated with the membrane for 1 hour at room temperature. The membrane was washed thrice (15 min. washes) with washing solution. Tween 20 was removed from the membrane with a single 15 min. wash in TBS. Bands

were detected by incubating the membrane with BCIP/NBT (Gibco BRL, Life Technologies, Inc., Gaithersburg, MD) in alkaline phosphatase substrate buffer (100 mM Tris-HCL, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) for 30 min. to 2 hours at room temperature. Once dark purple/blue bands appeared, the reaction was stopped by washing the membrane in distilled water and drying at room temperature.

Indirect Immunofluorescence. Samples of RP19 cells infected with HEV were dropped onto a glass slide and allowed to air dry. Chicken embryo fibroblasts (CEFs) were grown into a monolayer on glass coverslips. The CEF cultures were infected with recombinant FPV constructs at an multiplicity of infection (moi) of 0.6. Coverslips were harvested when lytic plaques were observed. When coverslips were harvested they were washed in PBS, fixed for 2 min. in ice cold acetone, and air dried. Fixed coverslips and slides were stored at -20 C for later use.

Indirect fluorescent antibody tests were performed as previously published (Fasina and Fabricant, 1982). Briefly, fixed coverslips or slides were wetted with PBS. The fixed samples were incubated with anti-native hexon MAb (monoclonal antibody) (kindly provided by Dr. Lucy Lee, Avian Disease and Oncology Laboratory, East Lansing, MI) diluted 1:100

in PBS for 30 min. at 37 C in a humidified incubator. Coverslips or slides were rinsed 15 min. in PBS then incubated for 30 min. with either goat anti-mouse fluorescein isothiocyanate (FITC)-conjugated goat anti- mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Infected cells were visualized using a dark-field microscope with UV (ploem) illumination.

Confocal microscopy. Confocal images were made with a laser scanning confocal microscope using an argon 488 nm line beam (Carl Zeiss, Inc.).

Immunoprecipitation. The positive control, HEV in RP19 cells, was grown as previously described. CEFs were grown to confluency in LM, 4% calf serum, penicillin/streptomycin and amphotericin B and infected with recombinant fowlpox virus (rFPV) at an moi of 5. Sixteen to twenty hours post infection (pi) (48 hours pi for positive control), the infected plates were incubated for 5 hours with methionine free RPMI medium 1640 (Gibco BRL, Life Technologies, Inc., Gaithersburg, MD). ^{35}S Methionine (New England Nuclear, Life Science Products, Boston, MA) was added and incubated for another 5 hours (12 hours for positive control). Cells were washed twice in PBS and lysed in lysis buffer (150 mM NaCl, 1% sodium

deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mM Tris-HCl [pH 7.5] in water).

Immunoprecipitation was performed in the following way. Sepharose protein A (Pharmacia-Biotech, Uppsala, Sweden) was prewashed with normal mouse serum for 3 hours. Antibody was incubated for 3 hours with Sepharose protein A. Cell lysate was added to Sepharose protein A and incubated for 3 hours. Proteins were precipitated when pre-washed antibodies were added to the lysate and Sepharose protein A. After 3 hours incubation on ice, 40 µl of SDS-Page loading buffer (1% SDS, 50 mM Tris-HCl [pH 6.8], 10% glycerol, 2.5% diethylthreotal, 0.01% phenol red in water) was added, the sample was boiled for 5 min., centrifuged at 10,000 x g for 5 min. and electrophoresed on an 8% SDS-Page gel. Electrophoresis was performed at 180 volts in running buffer (0.025 M Tris, 0.019 M glycine, 0.01% SDS in water).

The gel was fixed in destain solution for 45 min. and then incubated for 45 min. in dimethyl sulfoxide (DMSO), 45 min. in 2, 5-diphenyl oxazole (Sigma Chemical Co., St. Louis, MO) in DMSO, and washed for 10 min. in tap water. The gel was dried on filter paper with a Speed gel SG 200 gel

dryer (Savant, Farmingdale, NY) and exposed to film for 12-48 hours at -70 C.

Antibody ELISAs. HEV antibody was quantitated using a double sandwich antigen capture ELISA test as previously described (Nazerian et al., 1990). Briefly, 96-well Immulon I, flat bottomed plates (Dynatech Laboratories, Inc., Chantilly, VA) were incubated with anti-native hexon MAb (kindly provided by Dr. Lucy Lee, Avian Disease and Oncology Laboratory, East Lansing, MI) diluted 1:1000 in carbonate coating buffer (22 mM Na_2CO_3, 22 mM $NaHCO_3$ [pH 9.6]) for 48 hours at 4 C. The plates were washed twice with ELISA wash buffer (PBS with 0.1% Tween 80), air dried and stored at 4 C until used. Plates were blocked with blocking buffer (5% nonfat dry milk in PBS) for 1 hour at 41 C in a humidified incubator. The blocking buffer was removed and HEV antigen (virulent HEV grown in RP19 cells, sonicated and diluted at 5×10^5 cells/100 µl of blocking buffer) was added to each well and incubated overnight at 4 C. The plates were washed 3 times with wash buffer. Test sera were added to the first well at a dilution of 1:10 in blocking buffer. Serial 1:2 dilutions were made in subsequent wells in each row. The plates were incubated 1 hour at 37 C in a humidified incubator and then washed 3

times with ELISA wash buffer. Goat anti-turkey IgG labeled with horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was diluted 120 ng/ml in blocking buffer and added to each well. The plates were incubated 1 hour at 37 C in a humidified incubator and then washed 3 times with wash buffer. Phosphate buffer (0.2 M) , 0.8 mg/ml 5-amino salicylic acid (Sigma Chemical Co., St. Louis, MO), and 0.006% hydrogen peroxide was added to each well and the plates allowed to develop in the dark at room temperature for 2 to 6 hours, until color was fully developed. Plates were read on an automatic ELISA reader.

Humoral response to FPV-@X100, FPV-@100X and FPV-X100.

Chickens. Specific pathogen free (SPF) Line 15 X 7 chickens from the Avian Disease and Oncology Laboratory were used (Stone, 1975). Chickens were maintained in positive pressure isolators and given standard chicken ration and water *ad libitum*. Each group consisted of 13 chickens.

Chickens were raised in isolation until 4 weeks of age when they were inoculated with 10^5 pfu of FPV-@X100, FPV-@100X, or FPV-X100 via wing web stab or they were not inoculated. Positive control chickens were orally inoculated with 10^7 TCID virulent HEV at 5 weeks of age.

Chickens given the rFPVs were checked for fowlpox virus takes in the wing web 4-6 days post inoculation. Chickens were bled at 4 weeks of age, 5 weeks of age, and 6 weeks of age. Blood was allowed to clot, sera collected, and the sera tested for HEV antibodies with ELISA.

Turkeys. Turkeys were obtained from one of two sources of SPF turkeys either the Ohio Agricultural Research and Development Center, Ohio State University in Wooster, Ohio (trial 1) or the National Animal Disease Center in Ames, Iowa (trial 2). The total number of turkeys in a hatch were divided into 5 experimental groups: negative control (uninoculated), positive control (inoculated orally with a dose of the commercial HEV vaccine), FPV-X inoculated, FPV-@100 inoculated, and FPV-@X100 inoculated. These groups are shown in Table 4. Turkeys were maintained in positive pressure isolators and given standard turkey ration and water *ad libitum*.

Turkeys were raised in isolation until 4 weeks of age when they were inoculated with 10^5 FPV-X, FPV-@100, or FPV-@X100 via wing web inoculation, a single dose of HEV commercial vaccine (Oralvax HE, Schering-Plough Animal Health, Omaha, NE) given *per os*, or not inoculated. Turkeys given rFPVs were checked for fowlpox virus takes in

the wing web 4-6 days post inoculation. Turkeys were bled prior to inoculation and every 4 days after inoculation for 4 weeks and then once 1-2 weeks later. Blood was allowed to clot, sera collected and tested for HEV antibodies with ELISA.

Statistical analysis was done with the analysis tool pack of Microsoft Excel 5.0.

Results:

Determination of the presence of hexon and 100 kD folding protein genes in FPV recombinants. The Southern hybridization results (Figures 9 and 10) confirm the presence of the hexon and 100 kD folding protein genes in the expected recombinant FPVs. In addition, the digestion patterns are as predicted and confirm both the orientation of these genes and that there have been no significant mutations in the course of making these recombinants.

Expression of the nascent hexon and 100 kD folding proteins. Cytoplasmic proteins from RP19 cells and RP19 cells infected with HEV were extracted in order to increase the concentration of nascent proteins relative to the concentration of native hexon. Antibodies against both the

100 kD folding protein and the nascent hexon protein were detected in the sera of turkeys inoculated with the FPV-X and FPV-@100 recombinants. Nascent hexon protein and the 100 kD folding protein were detected in recombinants containing both the hexon and 100 kD folding protein genes. Both the nascent hexon and the 100 kD folding protein are approximately 80 kD. The native hexon is approximately 97.4 kD. In the three samples in which the native hexon appears, HEV, FPV-@100X, and FPV-@X100, the nascent hexon and 100 kD folding protein are only faintly visible. No hexon or 100 kD folding protein antibodies were detected in sera from turkeys inoculated with FPV-lacZ (Figure 11). Results are summarized in Table 3.

Native hexon was detected with indirect immunofluorescence. Again, anti-native hexon monoclonal antibody was used to detect expression of the native hexon. Native hexon was detected in RP19 cells infected with HEV (Figure 12A), and in CEFs infected with FPV-@X100 (Figure 12B), and FPV-@100X (Figure 12C). No positive immunofluorescence was detected in CEFs infected with FPV-X, FPV-@100, FPV-X100, or in FPV-lacZ (not shown). Findings are summarized in Table 3.

Expression of the native hexon. The native hexon of HEV can be precipitated with anti-native hexon monoclonal antibody (Nazerian et al., 1991). In the positive control RP19s infected with HEV, a 97.4 kD band, the native hexon, was precipitated. In neither FPV-lacZ, FPV-X, or FPV-@100 was a 97.4 kD band precipitated with the anti-native hexon monoclonal antibody. In two of the rFPVs, FPV-@X100 and FPV-@100X, the native hexon was immunoprecipitated. Finally, in one rFPV, FPV-X100 and in the case of coinfection with FPV-X and FPV-@100 no native hexon was precipitated (Figure 13).

Comparison of humoral response to FPV-@X100, FPV-@100X, and FPV-X100. The anti-HEV humoral immune response to FPV-@X100 was significantly higher ($p \leq 0.05$) than the immune response to FPV-@100X in chickens 14 days pi (Figure 14) No significant ($p \leq 0.05$) development of anti-HEV antibodies was detected in chickens given FPV-X100. Based on these results, the FPV-@X100 recombinant was used for the remaining study.

Humoral immune response to rFPVs. No anti-HEV antibodies were detected in turkeys inoculated with FPV-X or FPV-@100. This result is not unexpected since neither nascent hexon nor the 100 kD folding

protein are a part of the mature adenoviral virion. HEV antibodies appeared 12 days pi in turkeys vaccinated with the commercial HEV vaccine in both experimental trials (Table 4). Anti-HEV antibodies were detected in turkeys inoculated with FPV-@X100 on day 8 pi in trial 1 and day 16 pi in trial 2. The titers elicited by the commercial HEV vaccine were significantly higher than those elicited by FPV-@X100 on days 24, 28, and 35-42 pi. Results are shown in Table 4.

Discussion

The native hexon protein can be expressed in a vectored system by co-expression of the hexon and 100 kD folding protein. The expression of the native hexon in fowlpox virus (FPV) constructs required that the genes were cloned head to tail. Both the FPV-@X100 and FPV-@100X constructs expressed detectable levels of native hexon. However, the FPV-X100 construct did not. There may have been different levels of expression in the three constructs both *in vitro* and *in vivo*. However, since the same fowlpox virus promoters were used in all constructs and each of the rFPVs seemed to replicate to the same degree *in vitro* (as observed in cell culture) and *in vivo* (deduced from observing wing web takes after rFPV

inoculation) this is unlikely. It is also possible that a mutation in one or more of the cloned genes was introduced in the course of generating the rFPVs. Identical, cloned genes were used in each of the constructs and nascent proteins were expressed by other constructs (Figure 11) making this explanation unlikely. The final possibility is that the hexon and 100 kD folding protein have some yet undetermined mechanism for assembly which places constraints on their expression in a vectored system.

The 100 kD folding protein interacts with complete, newly synthesized hexon polypeptides but is not found in the mature virion (Grütter and Franklin, 1974, Cepko and Sharp, 1983, van Oostrum and Burnett, 1985). Virtually all of the hexon polypeptide bound to the 100 kD folding protein is destroyed by trypsin and therefore is not in the native conformation (Cepko and Sharp, 1982). The hexon polypeptide and 100 kD folding protein transiently associate on the polyribosomes during translation and remain as a complex in the cytoplasm (Cepko and Sharp, 1982). The hexon is formed at the time when the hexon polypeptides are released from their complex with the 100 kD folding protein. The 100 kD folding protein-hexon polypeptide complex thus plays a major role in hexon assembly and

may actually direct the folding of the hexon monomers into the trimeric, native conformation (Cepko and Sharp, 1983).

The anti-HEV humoral immune response elicited in turkeys inoculated with rFPVs expressing the native hexon reflects the fact that the hexon is the major viral protein in the virion and is the major antigenic component. The α antigen of adenoviruses is associated with the internal surface of the hexon, except in bovine and CELO virus which lack this antigenic determinant (Monreal, 1992). Hexons also carry the ε antigenic determinant, the type specific antigen, on the external surface of the capsid (Norrby and Wadell, 1969, Willcox and Mautner, 1976a, Willcox and Mautner, 1976b, Toogood et al., 1992). In its native form, hexon protein inoculated into turkey poults will protect them from disease after challenge with virulent HEV (van den Hurk and van Drunen Littel-van den Hurk, 1993). In addition, anti-hexon monoclonal antibodies are neutralizing for virulent HEV *in vitro* (Nazerian et al., 1991, van den Hurk and van Drunen Littel-van den Hurk, 1988) and are protective for turkeys challenged with virulent HEV (van den Hurk and van Drunen Littel-van den Hurk, 1993). Although the commercial HEV vaccine produces higher and longer lasting antibody titers than the rFPV, there could be significant practical

advantages to a rFPV vaccine for HEV in the poultry industry. For instance, a rFPV vaccine for HEV would potentially circumvent the immunosuppression observed relative to the use of the commercial HEV vaccine. In future experiments, the ability of the FPV-@X100 recombinant virus to protect turkeys from challenge with virulent HEV will be tested.

LEGENDS

FIGURE 8. Recombinant FPV constructs used. The direction of transcription, 5' to 3', is shown with arrows.

FIGURE 9. Southern hybridization of hexon DNA probe to digested DNA of rFPVs. Groups of 3 lanes: 1, FPV-lacZ; 2, FPV-X; 3, FPV-@100; 4, FPV-X100; 5, FPV-@X100; 6, FPV-@100X. Digests are ordered *BamHI*, *EcoRI*, and *HindIII* for each sample.

FIGURE 10. Southern hybridization of 100 kD folding protein DNA probe to digested DNA of rFPVs. Groups of 3 lanes: 1, FPV-lacZ; 2, FPV-X; 3, FPV-@100; 4, FPV-X100; 5, FPV-@X100; 6, FPV-@100X. Digests are ordered *BamHI*, *EcoRI*, and *HindIII* for each sample.

FIGURE 11. Western blot of cytoplasmic proteins from HEV-infected RP19 cells and uninfected RP19 cells. Groups of 2 lanes: 1, positive control polyclonal anti-HEV turkey serum; 2, negative control, anti-FPV-lacZ turkey serum; 3, anti-FPV-X turkey serum; 4, anti-FPV-@100 turkey serum; 5, anti-FPV-X100 chicken serum; 6, anti-FPV-@100X chicken serum; 7, anti-FPV-@X100 turkey serum. Cell lysates are ordered HEV infected RP19 cells on the left and uninfected RP19 cells on the right in each pair of lanes. Protein marker sizes are shown in kilodaltons at the left.

FIGURE 12. RP19 cells infected with HEV, indirect immunofluorescence assay using anti-native hexon monoclonal antibody.

FIGURE 13. CEFs infected with FPV-@100X, indirect immunofluorescence assay using anti-native hexon monoclonal antibody.

FIGURE 14. CEFs infected with FPV-@X100, indirect immunofluorescence assay using anti-native hexon monoclonal antibody.

FIGURE 15. Immunoprecipitation with anti-native hexon monoclonal antibody. Lanes: 1, HEV; 2, FPV-lacZ; 3, FPV-X; 4, FPV-@100; 5, FPV-X + FPV-@100; 6, FPV-X100; 7, FPV-@100X; 8, FPV-@X100. The native hexon precipitates at 97.4kD and is shown at right with arrow.

FIGURE 16. Comparison of humoral response to FPV-@X100, FPV-@100X and FPV-X100 in chickens. Graph represents the average log anti-HEV titer and error bars are shown at +/- one standard deviation.

TABLE 3. Summary of *in vitro* testing of rFPV constructs. ND = not done.

TABLE 4. Humoral immune response to rFPVs in turkeys in comparison to a commercial HEV vaccine, trials 1 and 2. Titers are expressed as log averages with the standard deviation given in parentheses. Different letters indicate statistically significant differences, ($p \leq 0.05$).

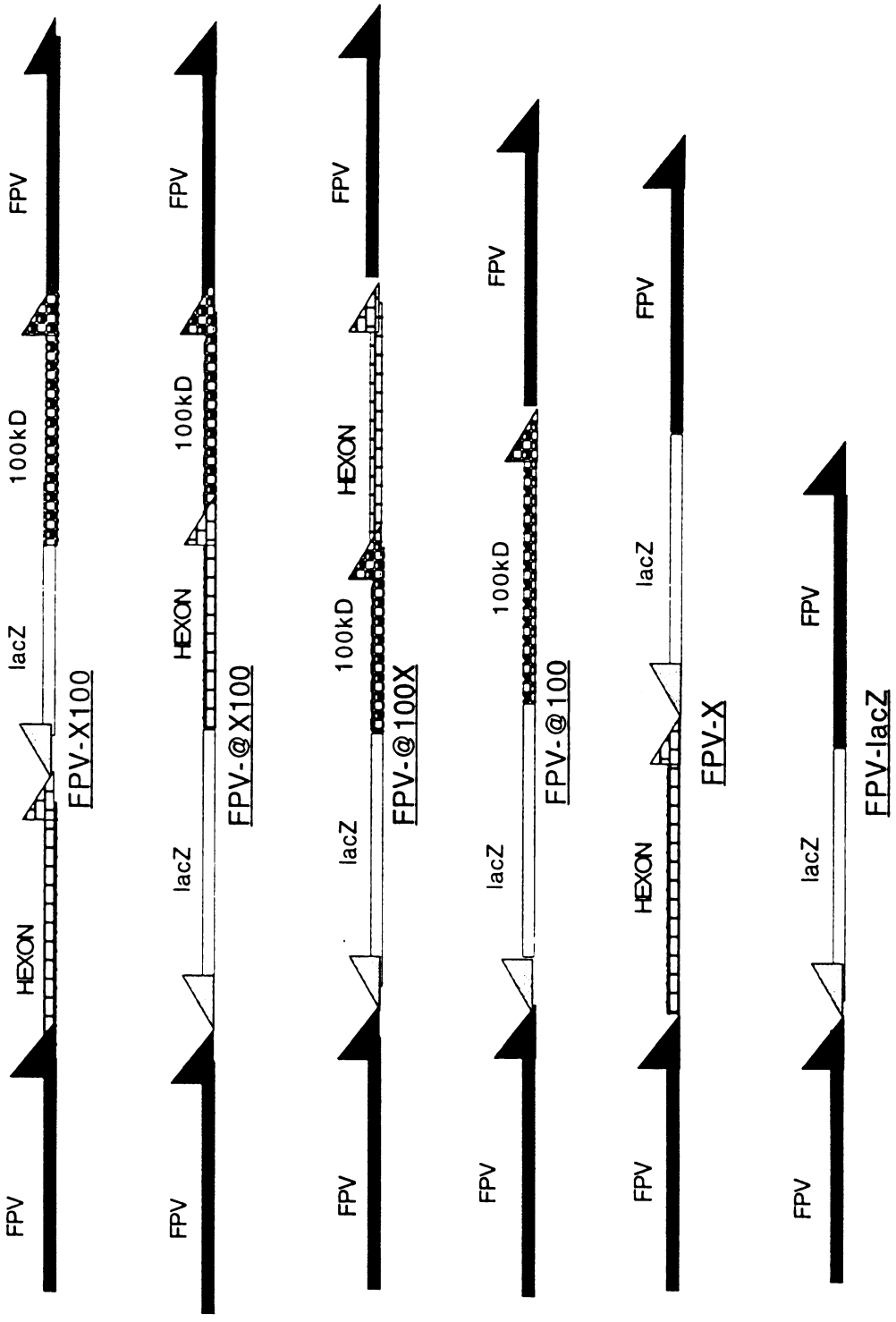

Figure 8. Recombinant FPV constructs used.

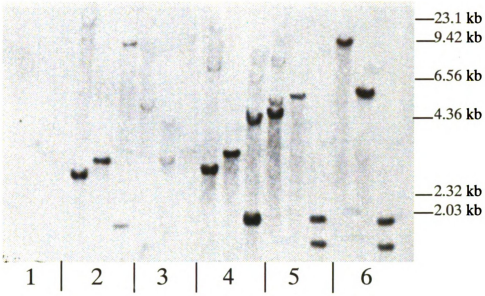

Figure 9. Southern hybridization of hexon DNA probe to digested DNA of rFPVs.

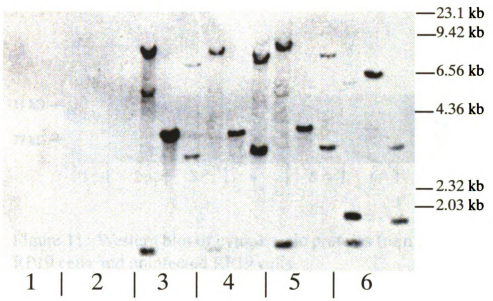

Figure 10. Southern hybridization of 100 kD folding protein DNA probe to digested DNA of rFPVs.

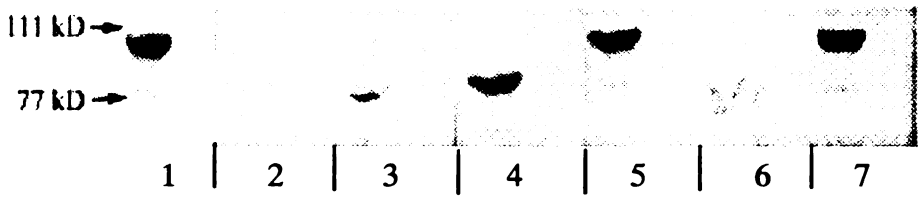

Figure 11. Western blot of cytoplasmic proteins from HEV-infected RP19 cells and uninfected RP19 cells.

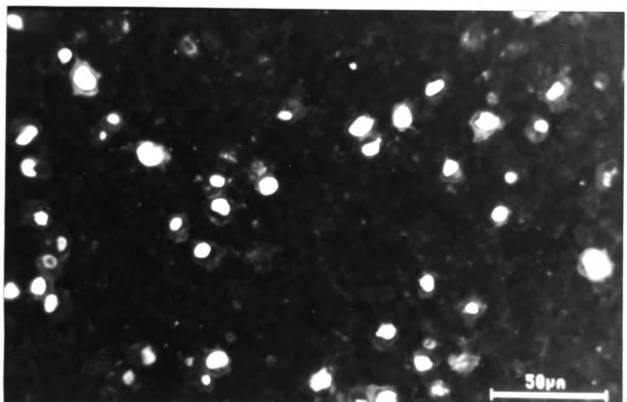

Figure 12. RP19 cells infected with HEV, indirect immunofluorescence assay using anti-native hexon monoclonal antibody.

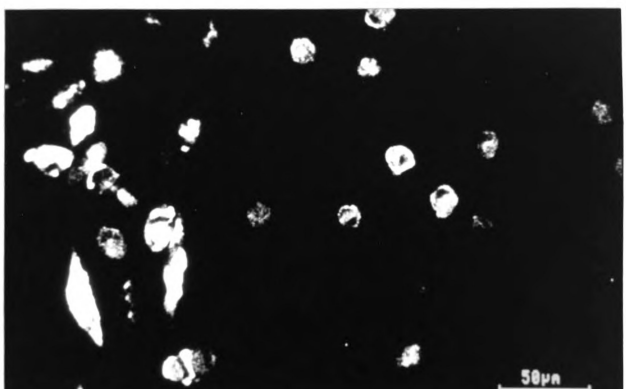

Figure 13. CEFs infected with FPV-@100X, indirect immunofluorescence assay using anti-native hexon monoclonal antibody.

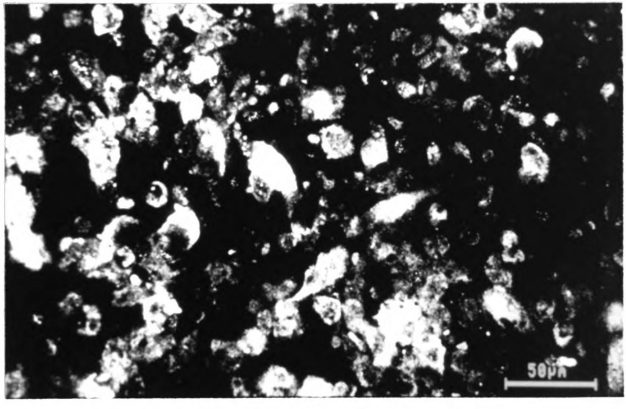

Figure 14. CEFs infected with FPV-@X100, indirect immunofluorescence assay using anti-native hexon monoclonal antibody.

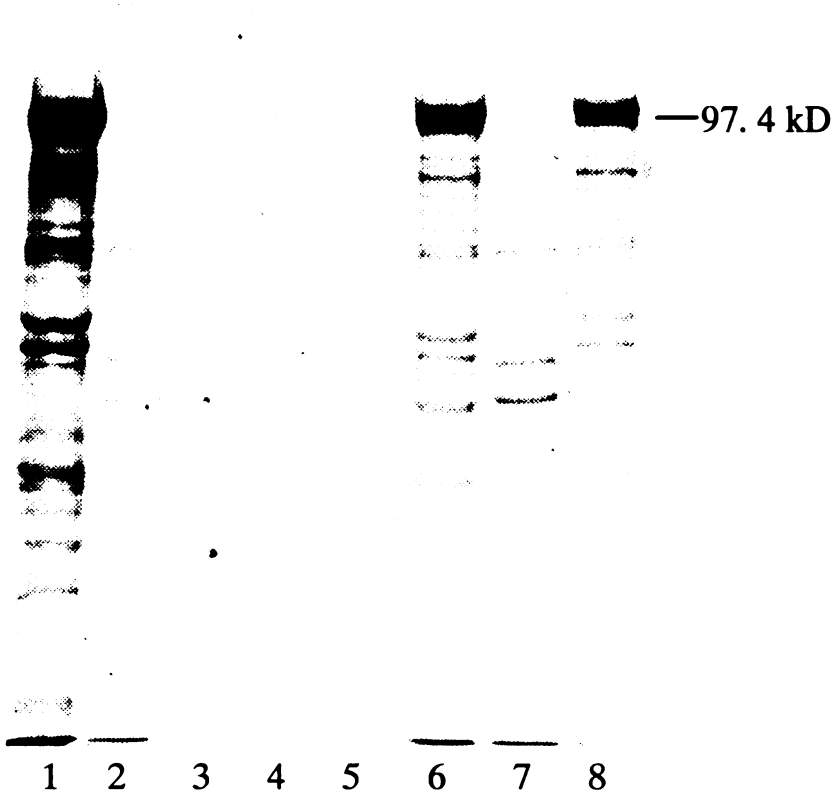

Figure 15. Immunoprecipitation with anti-native hexon monoclonal antbody.

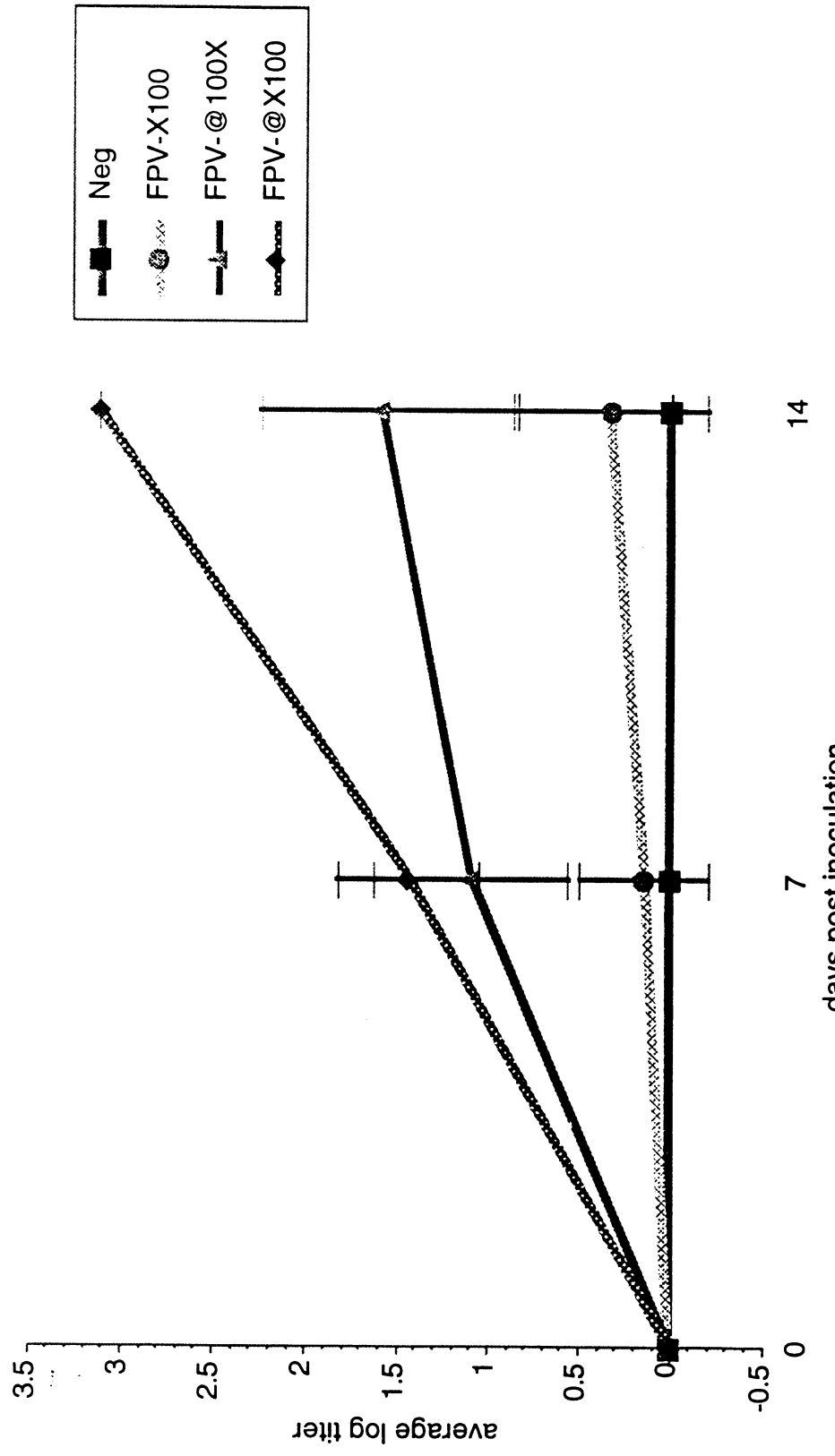

Figure 16. Comparison of humoral responses to rFPVs.

Table 3. Summary of *in vitro* testing of rFPV constructs.

Test	protein	FPV-lacZ	FPV-X	FPV-@100	FPV-X + FPV-@100	FPV-X100	FPV-@X100	FPV-@100X
Western blot	nascent hexon	–	+	–	ND	+	+	+
Western blot	nascent 100kD	–	–	+	ND	+	+	+
Immuno-precipitation	native hexon	–	–	–	–	–	+	+
Indirect immuno-fluorescence	native hexon	–	–	–	–	–	+	+
Humoral immune response	HEV	–	–	–	ND	–	+	+

Table 4. Humoral immune response to rFPVs in turkeys in comparison to a commercial HEV vaccine, trials 1 and 2.

Group	0 days pi		4 days pi		8 days pi		12 days pi		16 days pi		20 days pi		24 days pi		28 days pi		35 -42days pi	
Trial	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
neg	0.09 (0.30) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0.08 (0.28) a	0 (0) a	0 (0) a	0.09 (0.30) a	0.08 (0.28) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a
FPV-@X	0.09 (0.30) a	0 (0) a	0 (0) a	0.08 (0.29) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a
FPV-@100	0.09 (0.30) a	0.08 (0.28) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0.10 (0.36) a
FPV-@X100	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0 (0) a	1.65 (0.73) b	0.52 (0.87) b	2.00 (0.96) c	1.76 (0.66) c	2.70 (0.68) c	1.52 (0.81) c	3.24 (0.93) c,d	1.32 (0.90) b	1.93 (0.39) e	1.34 (0.71) e	1.57 (0.75) e	0 (0) a
vxHEV	0 (0) a	0 (0) a	0 (0) a	0 (0) a	0.33 (0.57) b	0 (0) a	0.91 (1.28) b	0 (0) a	1.65 (0.99) c	1.34 (1.32) c	3.30 (0.69) d	2.05 (1.58) c	2.78 (0.49) d	3.33 (0.59) d	2.78 (0.49) d	3.18 (0.92) d	3.71 (0.49) f	3.22 (1.12) d

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Chapter 4

PROTECTION OF TURKEYS FROM HEMORRHAGIC ENTERITIS WITH A RECOMBINANT FOWLPOX VIRUS EXPRESSING THE NATIVE HEXON OF HEMORRHAGIC ENTERITIS VIRUS

Abstract:

Hemorrhagic enteritis (HE) is an economically important disease of turkeys. It is caused by a type II aviadenovirus, hemorrhagic enteritis virus (HEV). The vaccines currently available to the commercial poultry producer are highly effective in preventing disease outbreaks, however, they are immunosuppressive. A recombinant fowlpox virus (rFPV) expressing the native hexon of HEV has been shown to induce an anti-HEV humoral immune response in turkeys (Chapter 3). In this study, a rFPV expressing the native hexon of HEV was compared to a commercial HEV vaccine (vxHEV) for its ability to protect turkeys from virulent HEV challenge. Complete protection from the intestinal lesions of HE was achieved in experimental groups vaccinated with either the rFPV or the vxHEV. Lymphocyte stimulation was measured in turkeys inoculated

with rFPV, vxHEV, a sublethal dose of HEV, or not inoculated. Immunodepression in turkeys given the rFPV was not significantly different from the variation observed in uninoculated turkeys.

Introduction

Hemorrhagic enteritis (HE) of turkeys is an economically important disease of turkeys characterized by hemorrhagic and necrotic intestinal mucosae especially severe in, but not confined to, the duodenum. Rapid death is common with dead turkeys often having full crops and gizzards (Domermuth and Gross, 1991, Saunders et al., 1993, McFerran et al., 1997). Flock mortality may reach 60% through the course of the disease (McFerran et al., 1997). In addition to this acute aspect of the disease, HEV causes a long lasting immunosuppression (Nagaraja, et al., 1982a, Nagaraja, et al., 1982b) which prevents turkeys from mounting effective immune responses against opportunistic infections (Larsen et al., 1985, Sponenberg, et al., 1985, Andral et al., 1985, Newberry, et al., 1993, van den Hurk et al., 1994, Pierson et al., 1996) and vaccine antigens (Nagaraja et al., 1985). Immunodepression may be insidious in onset and occur in the absence of the acute form of the disease (McFerran et al., 1997).

Hemorrhagic enteritis virus (HEV), a type II aviadenovirus, is the etiologic agent of HE (Carlson et al., 1974). Marble spleen disease virus (MSDV) and avian adenosplenomegaly virus (AASV) are also type II aviadenoviruses, antigenically indistinguishable from HEV (Domermuth et al., 1980). MSDV and AASV cause rapid death in their target species, pheasants and chickens respectively, but are of low pathogenicity in turkeys and other non-target species (McFerran et al., 1997).

Convalescent turkey serum administered to susceptible turkeys was the first method used to prevent outbreaks of HE (Domermuth et al., 1975). Gross lesions could be prevented with 0.5-1.0 ml of convalescent serum and intestinal lesions could be prevented with 0.1-0.25 ml of convalescent serum (Domermuth and Gross, 1975). Hyperimmune anti-HEV turkey serum was shown to prevent HE for up to 5 weeks post inoculation (pi) (Fadly and Nazerian, 1989). Later, turkey spleens with HEV and pheasant spleens with MSDV were processed, diluted 1:2 and administered to susceptible flocks in the drinking water (Domermuth et al., 1977). Recent evidence suggests that MSDV, long considered apathogenic for turkeys, is immunosuppressive (Sharma et al., 1992, Sharma, 1994). The administration of the spleens of HEV inoculated turkeys to susceptible birds

is also immunosuppressive and has the potential to introduce other problems as well.

A tissue culture attenuated HEV has been used extensively as a vaccine (Fadly et al., 1985). This vaccine is produced by passing virulent HEV in RP19 cells (Nazerian and Fadly, 1982, Fadly and Nazerian, 1984). The RP19 cell line is a Marek's disease virus (MDV) transformed turkey B-lymphocyte cell line which carries infectious MDV and can produce Marek's disease if inoculated into chickens (Nazerian et al., 1982). The tissue culture attenuated HEV vaccine has also been highly effective in preventing HE, although it too is immunosuppressive (Sharma, 1994).

In this work, a recombinant FPV (rFPV) expressing the native hexon of HEV is tested for its ability to protect turkey poults from challenge with virulent HEV. Previous reports demonstrate that an anti-HEV humoral immune response is induced in turkeys by vaccination with a rFPV expressing the native hexon of HEV (Chapter 3). Native hexon monoclonal antibodies are neutralizing (Nazerian et al., 1991, van den Hurk and van Drunen Littel-van den Hurk, 1993). Additionally, anti-hexon monoclonal antibodies inoculated into 6-week-old turkeys protected them from challenge with virulent HEV (van den Hurk and van Drunen Littel-van den

Hurk, 1993). Turkeys inoculated with native hexon protein were protected from both the lesions of HE and HEV infection (van den Hurk and van Drunen Littel-van den Hurk, 1993).

Both protection from infection and protection from the development of HE lesions after challenge with virulent HEV were measured in turkeys vaccinated with the rFPV expressing the native hexon of HEV and compared to a commercially available tissue culture attenuated HEV vaccine. The rFPV, the commercial HEV vaccine, and a non-lethal dose of virulent HEV were also compared and evaluated for their ability to cause immunodepression.

Materials and methods:

Protection study experimental design.

Turkeys. Broad-breasted white turkeys were obtained from Cuddy Farms, Strathroy, Ontario, Canada at one day of age. They were maintained in isolation and given standard turkey ration and *ad libidum* water. Turkeys were divided into four experimental groups: unvaccinated and unchallenged (negative control group); unvaccinated and challenged

(positive control group); vaccinated with the rFPV and challenged; vaccinated with the commercial HEV vaccine and challenged.

Turkeys were raised in isolation until 4 weeks of age when they were bled, sera collected and tested for HEV antibodies with ELISA. Turkeys were tested periodically for HEV antibodies until antibodies were no longer detectable. At 5-6 weeks of age (when turkeys were seronegative), poults were vaccinated with the recommended dose of vxHEV vaccine *per os* or with 10^5 pfu FPV-@X100 via wing web. Turkeys given FPV-@X100 were checked for fowlpox virus takes in the wing web 4-7 days post inoculation. One week following vaccination, turkeys in all but the negative control group were challenged with 10^6 TCID vHEV given *per os*. Six days following challenge, poults were euthanatized and necropsied. The same experimental protocol was repeated for two additional trials.

Immunosuppression study experimental design.

Turkeys. Broad-breasted white turkeys were obtained from Cuddy Farms, Strathroy, Ontario, Canada at one day of age. Turkeys were maintained in isolation and given standard turkey ration and *ad libidum* water. Turkeys available were divided into four experimental groups:

uninoculated (negative control); inoculated with vHEV (positive control); inoculated with vxHEV; inoculated with rFPV.

Turkeys were inoculated at 5-6 weeks of age with 10^5 pfu rFPV, a single dose of vxHEV given *per os*, or 10^3 TCID vHEV given *per os*. Blood was collected in heparin prior to inoculation, 6 days post inoculation and 17 days pi. Turkeys given the rFPV were checked for fowlpox virus takes in the wing web 6 days pi. The same experimental protocol was repeated for two additional trials.

Viruses. Construction and characterization of the rFPV expressing the native hexon has been described elsewhere (Chapter 3). Briefly, the hexon and 100 kD folding protein genes were cloned head to tail into a non-essential region of a FPV vector (Figure 15). Native hexon expression was detected using immunoprecipitation and indirect immunofluorescent antibody technique with an anti-native hexon MAb. This rFPV, FPV-@X100, when inoculated into both turkeys and chickens induced an anti-HEV humoral immune response. A commercial vaccine consisting of a tissue-culture attenuated strain of HEV was used in these trials (vxHEV; Oralvax HE, Schering-Plough Animal Health, Omaha, NE). The challenge virus, virulent HEV (vHEV) was originally obtained from C. H. Domermuth

(Virginia Polytechnic Institute) as spleen homogenates. The challenge virus was propagated in RP19 cells, harvested, and stored at -70 C for further use.

Antigen ELISAs. HEV antigen in spleens was quantitated by an antigen capture ELISA as previously described (Nazerian et al., 1986). Briefly, spleens were collected at necropsy and stored at -20C until use. Splenic tissues were homogenized by passage through a syringe and needle. The tissues were then diluted 1:3 (weight to volume) in ELISA wash buffer (Phosphate buffered saline [PBS] and 0.1% Tween 80).

Flat bottomed Immulon I 96-well plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated with anti-hexon monoclonal antibody (kindly provided by Dr. Lucy Lee, Avian Disease and Oncology Laboratory, East Lansing, MI) diluted 1:1000 in carbonate coating buffer (22 mM Na_2CO_3, 22 mM $NaHCO_3$ [pH 9.6]) for 48 hours at 4 C. Plates were washed 2 times with ELISA wash buffer. The wells were blocked with 5% non-fat dry milk in PBS (blocking buffer) and incubated at 37 C for one hour in a humidified incubator. Antigen was added and serially diluted. Plates were washed 3 times with ELISA wash buffer. Positive anti-HEV turkey serum, diluted in blocking buffer was added to each well and incubated at 37 C for 1 hour in a humidified incubator. Plates were washed 3 times with ELISA wash

buffer. Goat anti-turkey IgG labeled with horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was diluted 120 ng/ml in blocking buffer and added to each well. The plates were incubated 1 hour at 37 C in a humidified incubator and then washed 3 times with ELISA wash buffer. Phosphate buffer (0.2 M) , 0.8 mg/ml 5-amino salicylic acid (Sigma Chemical Co., St. Louis, MO), and 0.006% hydrogen peroxide was added to each well and the plates allowed to develop in the dark at room temperature for 2 to 6 hours, until color was fully developed. Plates were read on an automatic ELISA reader.

Antibody ELISAs. HEV antibody was quantitated using a double sandwich antigen capture ELISA test as previously described (Nazerian et al., 1991). Briefly, 96-well Immulon I, flat bottomed plates were coated with anti-hexon monoclonal antibody diluted 1:1000 in carbonate coating buffer for 48 hours at 4 C. The plates were washed twice with ELISA wash buffer, air dried and stored at 4 C until used. Plates were blocked with blocking buffer for 1 hour at 37 C in a humidified incubator. The blocking buffer was removed and HEV antigen (virulent HEV grown in RP19 cells, sonicated and diluted at 2.5 mg/ml in blocking buffer) was added to each well and incubated overnight at 4 C. The plates were washed 3 times with

ELISA wash buffer. Test sera were added to the first well at a dilution of 1:10 in blocking buffer. Serial 1:2 dilutions were made in subsequent wells in each row. The plates were incubated 1 hour at 37 C in a humidified incubator and then washed 3 times with ELISA wash buffer. Goat anti-turkey IgG labeled with horseradish peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was diluted 1:1000 in blocking buffer and 100 µl added to each well. The plates were incubated 1 hour at 37 C in a humidified incubator and then washed 3 times with ELISA wash buffer. Phosphate buffer (0.2 M), 0.8 mg/ml 5-amino salicylic acid (Sigma Chemical Co., St. Louis, MO), and 0.006% hydrogen peroxide was added to each well and the plates allowed to develop in the dark at room temperature for 2 to 6 hours, until color was fully developed. Plates were read on an automatic ELISA reader.

Lymphoblastogenesis. Fifteen ml whole blood was collected into 5 ml RPMI medium 1640 (Gibco BRL, Life Technologies, Gaithersburg, MD) containing 100 U/ml heparin sulfate. The blood was divided into two 15 ml conical tubes and centrifuged at 750 rpm for 15 min. The buffy coat was swirled with a glass pipette and collected into a sterile tube. Lymphocytes were washed 3 times in RPMI medium 1640, counted, and

resuspended to a concentration of $1x10^5$/100 µl. The lymphocytes were cultured in 96 well Microtest III tissue culture plates (Falcon, Becton Dickson, Franklin Lakes, NJ) in RPMI-1640 medium containing 0.05% chicken serum (Gibco BRL, Life Technologies, Grand Island NY), penicillin, streptomycin, and amphotericin B. Lymphocytes were stimulated with 0.4 µg/well of concanavalin A ([ConA]; Pharmacia-Biotech, Uppsala, Sweden) or 2 µg/well of phytohemaglutinin-P ([PHA-P]; Difco Laboratories, Detroit, MI). The lymphocyte cultures were incubated at 37 C in 5% CO_2 for 48 hours. One µCi of ^{3}H-thymidine in 50 µl of RPMI-1640 medium was added to each well and incubated for an additional 24 hours. At the end of the 72 hours of incubation, cells were harvested onto glass fiber filter paper using a semi-automatic cell harvester (Brandel, Rockville, MD). Filter paper discs were air dried overnight, then placed in 1 ml of scintillation fluid (Ecoscint, National Diagnostics, Atlanta, GA). Counts per minute (cpm) were measured for 1 min. using a liquid scintillation analyzer (Packard Tri-Carb 1500, Downers Grove, IL).

Histopathology. Splenic and duodenal tissues were collected in 10% neutral buffered formalin, processed routinely, and stained with hematoxylin and eosin.

Results:

Protection study. There were no intestinal lesions observed grossly or histologically in the groups vaccinated with either FPV-@X100 or vxHEV or in our negative control group. These findings indicate that 100% protection from the intestinal lesions of HEV was achieved with either the rFPV or the commercial HEV vaccine after challenge with virulent HEV. Of the total positive control turkeys, 56% showed the typical intestinal lesions of HE. Results are summarized in Table 5.

Infection with vHEV was defined as either a splenic antigen ELISA titer equal to or greater than 1:100 and/or the observation of adenovirus intranuclear inclusions in tissue sections of intestine or spleen. There was 57.1%, 0%, and 39.6% protection from infection in the vxHEV groups. 42.9%, 0%, and 49.8% protection from infection in the FPV-@X100 groups in each of the three trials respectively. The total protection from infection achieved in all three trials was 21.9% for the vxHEV vaccinated group and 32.8% for the rFPV vaccinated group. Results of the splenic antigen ELISAs are shown in Table 6. Results of the histological examination of spleens are shown in Table 7.

In an effort to compare challenge virus replication in vaccinated poults, the spleen to body weight ratios and antigen ELISA titers of turkeys vaccinated with FPV-@X100, and vxHEV were compared to the positive and negative control groups. Results are summarized in Tables 6 and 8. There was no significant difference ($p \leq 0.05$) between the two groups vaccinated with either FPV-@X100, vxHEV, or the positive control group. However, there was a significant difference ($p \leq 0.05$) between these groups and the negative control group.

Immunosuppression study. In 1 of 10 turkeys (10%) given the FPV-@X100 vaccine, immunodepression was measured with PHA and ConA stimulation on day 17 pi. In 2 of 10 turkeys (20%) given the FPV-@X100 vaccine, immunodepression with either PHA or ConA stimulation but not both was observed. A total of 3 of 10 turkeys (30%) were immunodepressed in this treatment group. Immunodepression was defined, for this study as a fall in the stimulation index from day 0 or 6 pi to day 17 pi of 50% or more. Immunodepression was measured in 4 of 9 turkeys (44.4%) given vxHEV measured by both PHA and ConA stimulation and in 4 of 9 turkeys (44.4%) measured by PHA stimulation alone. A total of 8 of 9 turkeys given vxHEV (88.9%) were immunodepressed. Among turkeys

inoculated with a low dose of virulent HEV, 4 of 11 turkeys (36.4%) were immunodepressed with both PHA and ConA stimulation and 1 of 11 turkeys (9.1%) with PHA alone. A total of 5 of 11 turkeys (45.6%) given virulent HEV were immunodepressed. No immunodepression was measured in 8 of 8 turkeys (0%) given no inoculum measured with both PHA and ConA and 1 of 8 turkeys (12.5%) was immunodepressed as measured with PHA stimulation alone. A total of 1 of 8 turkeys (12.5%) was immunodepressed in the negative control group. Results are summarized in Table 9.

Discussion:

The mechanism by which anti-hexon antibodies act to prevent hemorrhagic enteritis is not known. However, an analogy with Ad5, might provide some answers. In subgroup B and D human adenoviruses including Ad5, anti-hexon antibodies can block hemagglutination. This is because Ad5 has a very short fiber and anti-hexon antibodies attached to peripentonal hexons block the attachment of the fiber to its cellular receptor (Philipson, 1983). Like Ad5, the type II avian adenoviruses have short fibers (van den Hurk and van Drunen Littel-van den Hurk, 1988, Zhang et al., 1991). The avian adenoviruses are not hemagglutinating viruses,

however, peripentonal anti-hexon antibodies may prevent attachment of the virus to target host cells in a similar way. The penton and fiber have been implicated as proteins of importance in the entry of adenoviruses into target cells. In the case of HEV with a very short fiber and MSDV and AASV with no fibers, peripentonal anti-hexon antibodies could block the interaction of the penton and the fiber with cellular receptors.

Anti-hexon antibodies have also been shown to block adenovirus infection by preventing the pH dependent release of the adenoviral virion from the endosome (Varga et al., 1990). This theory more closely fits with the observed kinetics of anti-hexon antibodies in mammalian systems showing a single hexon antibody is required to neutralize the virus.

The enteric lesions of HE were prevented with a single wing web vaccination with a FPV recombinant expressing the native hexon of HEV after challenge with virulent HEV. However, HEV infection, as demonstrated by splenomegaly and the presence of HEV splenic antigen, was not prevented by vaccination with the recombinant vaccine. Based on these experiments, it was not determined whether or not vxHEV prevents infection since the challenge virulent HEV from the vaccine strain HEV cannot be differentiated with the methods used.

The large individual bird to bird variation observed in the immunodepression experiment is consistent with the use of outbred experimental animals (Dorey and Zighelboim, 1980). Although this may accurately represent the field situation, it leads to a confusing picture. The immunodepression observed in the vxHEV and virulent HEV treated groups was significantly greater than that observed in the rFPV or negative control groups. The immunodepression observed in the negative control and rFPV treated groups was statistically indistinguishable. From these results, it can be concluded that FPV-@X100 does not induce a statistically significant immunodepression. Further studies should be done to confirm these results.

Levels of anti-HEV antibodies required to prevent the intestinal lesions of HE are lower than the levels required to prevent HEV replication (Domermuth and Gross, 1975). Anti-hexon, and hence, anti-HEV antibody may prevent viral replication if present at high titers (van den Hurk and van Drunen Littel-van den Hurk, 1993). However, HEV replication in the spleen was not prevented with this rFPV. This most likely indicates that the titer of anti-HEV antibody elicited by the rFPV is sufficient to prevent intestinal lesions but is not great enough to prevent viral replication.

Currently there are two commonly used and readily available vaccine types for the prevention of HEV in turkeys in the United States. One is the commercially prepared tissue culture attenuated HEV vaccine and the other is the splenic origin vaccine. Both vaccines have significant drawbacks for use in commercial turkeys. As mentioned previously, both vaccines are immunosuppressive and can be problematic in commercial and breeder turkeys for this reason. In addition, the inoculation of turkeys with material from non-SPF turkeys, as is done with splenic origin vaccines, poses some questions of quality and purity. The FPV-@X100 construct offers some advantages to the currently available vaccines in these areas of concern as has been presented.

However, as with anything, these advantages are balanced by some drawbacks to the use of this rFPV as a vaccine. Not least among these potential complications is the difficulty in delivering a FPV or FPV-vectored vaccine to commercial poultry. Commercial turkeys are raised in large flocks and the labor required to catch and handle birds individually, is immense. For this reason, most producers prefer vaccines which can be given orally. Although some reports indicate that FPV given orally can protect chicks from challenge with FPV (Nagy et al., 1990), most suggest

that wing web administration is the most effective delivery system (Saini et al., 1990, Tripathy and Reed, 1997). In addition, recombinant FPVs (rFPVs) administered via wing-web stick or subcutaneously elicited antibodies against FPV and expressed foreign antigens (Beard et al., 1992, Boyle and Heine, 1994) but rFPVs administered intranasally, conjunctivally (Beard et al., 1992, Boyle and Heine, 1994), or in the drinking water (Beard et al., 1992) elicited no immune response neither against FPV nor against any expressed foreign antigen.

Vaccine delivery to large groups of birds is a problem for poultry producers, however, under certain circumstances, FPV vaccines are given in the wing web to commercial turkeys anyway. Commercial turkeys are vaccinated against FPV in areas and during times of the year where and when FPV outbreaks are common. Breeder turkeys are handled several times during their lives and are commonly given one or more FPV inoculations. The difficulty of handling large numbers of turkeys remains a problem, however, many turkeys are being handled anyway and FPV-vectored vaccines could be administered in place of FPV vaccines thus protecting them from both FPV and HEV.

The rFPV described may be a viable third choice for the commercial poultry producer for the prevention of HE in turkeys. Although the current vaccines provide excellent protection, they have significant drawbacks. This rFPV vaccine may circumvent the problems associated with the use of commercial and splenic origin HEV vaccines. Further testing of this vaccine under field conditions should be done to determine its efficacy and practicality for the turkey producer.

LEGENDS

TABLE 5. Numbers of individual turkeys with hemorrhagic enteritis after challenge per total in experimental group.

Group = experimental group

Number of turkeys with intestinal lesions/number in group

TABLE 6. Antigen ELISA results. Splenic antigen ELISA titers were analyzed with ANOVA. Values with the same letter following are not statistically different.

Group = experimental group

Number of turkeys with positive antigen ELISA/number in group

SD = standard deviation

TABLE 7. Intranuclear inclusion bodies in splenic and enteric tissues from experimental groups of turkeys.

Group = experimental group

Number of turkeys with inclusion bodies/number in group

TABLE 8. Splenomegaly in experimental groups of turkeys.

Group = experimental group

Number of turkeys with splenomegaly/number in group

SD = standard deviation

spl/bw = spleen/body weight

TABLE 9. Summary of results from all immunosuppression trials.

n = individual turkeys

no. dep. = number of individuals depressed greater than 50%

% dep. = percent of total individual turkeys depressed

Table 5. Numbers of individual turkeys with hemorrhagic enteritis after challenge per total in experimental group.

Group	Trial 1	Trial 2	Trial 3	total	% total
unvac/unchal	0/6	0/2	0/32	0/40	0%
unvac/chal	5/6	3/6	16/31	24/43	56%
FPV@X100/chal	0/7	0/6	0/26	0/40	0%
vxHEV/chal	0/7	0/6	0/27	0/39	0%

Table 6. Antigen ELISA results.

Group	Antigen ELISA ≥1:100					log Ave. titer (SD)			
	Trial 1	Trial 2	Trial 3	Total	% total	Trial 1	Trial 2	Trial 3	Total
unvac/unchal	0/6	0/2	0/32	0/40	0%	0.23 (0.57)	0 (0)	0.05 (0.25)	0.07 (0.30) a
unvac/chal	6/6	3/6	10/31	19/43	44.2%	2.92 (1.43)	1.60 (1.79)	1.17 (1.04)	1.65 (1.33) b
FPV@X100/ chal	4/7	4/6	4/26	12/40	30%	1.70 (1.87)	1.89 (1.58)	0.86 (1.03)	1.21 (1.33) b
vxHEV/chal	3/7	6/6	5/27	14/39	35.9%	1.17 (1.81)	3.05 (0.76)	0.83 (1.02)	1.28 (1.39) b

Table 7. Intranuclear inclusion bodies in splenic and enteric tissues from experimental groups of turkeys.

Group	splenic inclusions				enteric inclusions			
	Trial 1	Trial 2	Trial 3	total	Trial 1	Trial 2	Trial 3	total
unvac/unchal	0/6	0/2	0/32	0/40	0/6	0/2	0/32	0/40
unvac/chal	3/6	2/6	19/31	24/43	0/6	0/6	3/31	3/43
FPV@X100/ chal	4/7	3/6	8/26	15/40	0/7	1/6	0/26	1/40
vxHEV/chal	3/7	4/6	10/27	17/39	0/7	4/6	2/27	6/39

Table 8. Splenomegaly in experimental groups of turkeys.

Group	turkeys with spl/bw ratios over 1.5					Ave. spl/bw ratio (SD)			
	Trial 1	Trial 2	Trial 3	total	% total	Trial 1	Trial 2	Trial 3	total
unvac/unchal	0/6	1/2	0/32	1/40	2.5%	1.09 (0.23)	1.48 (0.12)	0.97 (0.16)	1.01 (0.20)
unvac/chal	4/6	2/6	26/31	32/43	74.4%	1.70 (0.68)	1.5 (0.5)	2.05 (0.64)	1.96 (0.66)
FPV@X100/ chal	4/7	5/6	25/26	34/40	85%	2.56 (1.5)	2.76 (0.94)	2.16 (0.55)	2.30 (0.83)
vxHEV/chal	3/7	6/6	21/27	30/39	76.9%	1.33 (1.01)	2.71 (0.76)	2.16 (0.72)	2.12 (0.84)

Table 9. Summary of results from all immunosuppression trials.

Trial	Treatment	n	PHA no. dep.	% dep.	ConA no. dep.	% dep.	total % dep.
1	negative	2	0		0		0%
2		3	0		0		0%
3		3	1		0		33.3%
TOTAL		8	1	12.5%	0	0%	12.5%
1	FPV@X100	2	0		1		50%
2		4	1		0		25%
3		4	1		1		25%
TOTAL		10	2	20%	2	20%	30.0%
1	vxHEV	2	2		1		100%
2		4	4		1		100%
3		3	2		2		66.7%
TOTAL		9	8	88.9%	4	44.4%	88.9%
1	HEV	2	2		1		100%
2		4	3		3		75%
3		5	0		0		0%
TOTAL		11	5	45.5%	4	36.4%	45.5%

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SUMMARY OF RESEARCH FINDINGS

1. A phylogenetic analysis of adenoviruses which includes hemorrhagic enteritis virus, a type II aviadenovirus, gives a new perspective to the classification of aviadenoviruses. The three serogroups of aviadenoviruses are only distantly related to each other. However, they are more closely related to each other than to mastadenoviruses. The exception is the aviadenovirus-like ovine adenovirus 287 which is phylogenetically close to egg drop syndrome-76 virus.

2. The native hexon was expressed in a recombinant fowlpox virus vector. Native hexon expression required co-expression of both the hexon and 100 kD folding protein in a single fowlpox virus vector. Additionally, native hexon expression required that the hexon and 100 kD folding protein genes be cloned head to tail.

3. A fowlpox virus vector expressing the native hexon of hemorrhagic enteritis virus elicited an anti-hemorrhagic enteritis virus humoral immune response in turkeys. No anti-hemorrhagic enteritis virus

humoral immune response was detected in turkeys inoculated with fowlpox virus vectors expressing the nascent hexon alone or the 100 kD folding protein alone. These results indicate that an anti-hemorrhagic enteritis virus humoral immune response can be elicited by the native hexon produced by the co-expression of the hexon and 100 kD folding protein but not by either protein alone.

4. A fowlpox virus vector expressing the native hexon of hemorrhagic enteritis virus was shown to protect turkeys from the enteric lesions of hemorrhagic enteritis after challenge. However, there was no protection from infection with hemorrhagic enteritis virus after challenge as determined by the appearance of viral inclusions or antigen in spleens and splenomegaly. These results are identical to those observed in turkeys vaccinated with a tissue culture attenuated hemorrhagic enteritis virus commercial vaccine and challenged with virulent hemorrhagic enteritis virus. These results suggest that this fowlpox virus expressing the native hexon of hemorrhagic enteritis virus might make a suitable vaccine.

5. Cell-mediated immune status was compared in turkeys inoculated with a dose of the vaccine strain of hemorrhagic enteritis virus, virulent hemorrhagic enteritis virus, a fowlpox virus recombinant expressing the

native hexon of hemorrhagic enteritis virus, or uninoculated. No statistically significant immunodepression was measured in turkeys vaccinated with the fowlpox virus recombinant, 17 days post inoculation when compared to the uninoculated negative control group. Individual bird to bird variation prevented making any statistically significant conclusions from the vaccine or virulent strain of hemorrhagic enteritis virus inoculated groups.

VITA

The author was born in Santa Barbara, California in 1961 and lived in Goleta, California, a suburb of Santa Barbara, until 1967. From 1967-1969, she and her family lived in Hanford, California where the author attended kindergarten and first grade at Lee Richmond Elementary School. In 1969, the author moved with her family to American Samoa where they lived for two years. The author attended second and third grade at Fia Iloa Elementary School. In 1971, the author and her family moved to Palm Desert, California. She completed fourth and fifth grade at Lincoln Elementary School, attended sixth through eighth grade at Palm Desert Middle School, and ninth grade at Indio High School. In 1977, the author moved to Crawfordsville, Indiana where she completed high school at Crawfordsville High School.

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