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Expression of mutated forms of the bovine coronavirus hemagglutinin-esterase protein in stably transformed mammalian cells

Joanne L. Maki

To the Graduate Council:

I am submitting herewith a thesis written by Joanne L. Maki entitled "Expression of mutated forms of the bovine coronavirus hemagglutinin-esterase protein in stably transformed mammalian cells." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Comparative and Experimental Medicine.

David A. Brian, Major Professor

We have read this thesis and recommend its acceptance:

Karla Matteson, Leon Potgieter

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Dean of the Graduate School

EXPRESSION OF MUTATED FORMS OF THE BOVINE CORONAVIRUS HEMAGGLUTININ-ESTERASE PROTEIN IN STABLY TRANSFORMED MAMMALIAN CELLS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Joanne L. Maki

May 1991

I dedicate my thesis to the memory of:

Dr. Gordon Phillips, V.M.D.

As a mentor, he encouraged my discovery
of science and veterinary medicine; as a employer, he
gave me confidence; as a friend, his humor and compassion
made it all enjoyable.

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ABSTRACT

The hemagglutinin-esterase protein is one of three integral membrane glycoproteins on the surface envelope of the hemagglutinating coronaviruses. It is found as a140 kD homodimer composed of disulfide linked monomers of 65 kD. This glycoprotein was first identified and characterized in our laboratory during studies on the bovine coronavirus. The hemagglutinin-esterase gene was recently cloned from the bovine coronavirus RNA genome, sequenced, and transiently expressed in vitro in our laboratory.

To develop an in vitro system to study the synthesis and expression of viral glycoproteins, the hemagglutinin-esterase gene was subcloned into an expression vector, transfected into recipient mammalian cells, and cell lines containing the gene were established. Stably transfected cells were shown to contain the integrated hemagglutinin-esterase gene as determined by polymerase chain reaction amplification of specific gene sequences. Polymerase chain reaction products were shown to be viral specific by Southern blots probed with hemagglutinin-esterase gene specific oligonucleotides.

Despite stable transfection and propagation of clonally selected cell lines, the hemagglutinin-esterase protein could not be detected as a 140 kD dimer that would reduce to a 65 kD monomer. Abnormal products of the hemagglutinin-esterase gene were detected by immunoprecipitation with hemagglutinin-esterase specific polyclonal and monoclonal antiserums. These suspected mutated forms of the hemagglutinin-esterase protein were approximately 100 kD and 60 kD in size and did not reduce to smaller species. Polyclonal antiserum directed against the hemagglutinin-esterase protein (gp65) detected low levels of hemagglutinin-esterase protein in the cytoplasm of transfected cells by indirect immunofluorescence. Transfected cells expressing these mutated forms did not exhibit hemadsorption.

Hypotheses explaining how mutated forms of the HE protein might have

arisen during stable transformation are put forth: (1) Necessary viral gene sequences required for the correct transcription of the hemagglutinin-esterase gene may have been deleted during integration of the plasmid DNA into the cell genome during replication; (2) Expression of the complete hemmagglutinin-esterase gene may have been toxic to the host cell resulting in the selection of only mutated forms of the gene.

One striking observation was that uninfected human rectal tumor cells, one of the host cells used in this expression system, contain a disulfide-linked dimeric protein of 200kD (reducible to subunits of 95 kD) that is specifically immunoprecipitated with one of the hemagglutinin-esterase specific monoclonal antibodies. Since this cellular protein and the hemagglutinin-esterase protein are both disulfide-linked dimers, the intriguing possibility arises that the hemagglutinin-esterase protein and the immunoprecipitated cellular protein are evolutionarily related. Taken one step further, perhaps this coronavirus surface envelope glycoprotein originated from a cellular protein. Such a striking structural homology between a viral and cellular protein suggests there may be mimicry of a cell surface protein by the hemagglutinin-esterase protein during infection leading to pathological consequences.

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INTRODUCTION

The Hemagglutinin-Esterase Protein of Bovine Coronavirus

The hemagglutinin-esterase (HE) protein is found on the surface envelope of six different species of coronaviruses. They are the bovine coronavirus (BCV; King and Brian, 1982), the human coronavirus OC43 (Hogue and Brian, 1986), the porcine hemagglutinating encephalomyelitis virus (Callebaut and Pensaert, 1980), the turkey coronavirus (Dea and Tijssen, 1988), the mouse hepatitis virus strain JHM (Taguchi et al., 1986), and the diarrheal virus of infant mice (Sugiyama, et al., 1980; Sugiyama, et al., 1986).

The HE protein of the bovine coronavirus was the first HE protein to be identified and characterized (King and Brian, 1982; King et al., 1985). On the virion, the HE glycoprotein exists as a homodimer (gp140) composed of two identical subunits (gp65) covalently linked by disulfide bonds. The dimer is heavily glycosylated and contains numerous asparagine-linked sugar residues (N-linkages; Deregt et al., 1987b; Hogue et al., 1989). The sequence of the HE gene is known and in vitro expression studies have shown it to be a Type I glycoprotein (Kienzle et al., 1990). The HE protein is a unique viral glycoprotein in that it is thought to assemble by the rapid disulfide linkage of two identical subunits during synthesis rather than by cleavage of a precursor protein.

The HE protein is one of three major structural glycoproteins found on the surface envelope of coronaviruses (Holmes, 1985). This glycoprotein imparts hemagglutinating and esterase activities to the virus and probably acts as a major attachment protein during viral infection (King et al., 1985). Antibodies directed against the HE protein are capable of neutralizing infectious virus both in vitro and in vivo (Deregt et al., 1987a; Deregt et al., 1989). These data suggest that the HE protein is a strongly antigenic protein that has required functions during the

infection process. Therefore, the HE protein may be involved in stimulating the host's immune response during coronavirus infection and thus, be an important immunogen for vaccine development.

The Rationale Behind Expressing the Bovine Coronavirus Hemagglutinin-Esterase Protein in Mammalian Cells

The bovine coronavirus is an economically important virus that causes severe gastroenteritis in neonatal calves (House, 1978; Mebus, et al., 1973). Approximately 15 to 25 percent of susceptible calves may exhibit clinical signs during an outbreak of coronaviral enteritis and 5 to 10 percent may die (Langpap et al., 1979). However, deaths due to coronavirus infections may be significantly higher in cases of secondary viral or bacterial infections, such as E. coli enterotoxemia (House, 1978).

Although coronavirus infections are not commonly associated with disease in adult cattle, close to 100% of bovine sera and lacteal secretions surveyed contain antibodies to BCV (Rodak, et al., 1982). Coronaviruses have been thought to cause isolated epizootics of diarrhea in cattle and recently, infectious coronavirus has been isolated from adult cattle exhibiting winter dysentery, a severe bloody diarrhea (Takahashi et al., 1980; Benfield and Saif, 1990).

Even though the clinical presentation and pathogenesis of BCV have been described (Sharpee et al., 1976), much remains unknown about this mammalian RNA virus. Studying the complex interactions of a virus and its host in detail is difficult, if not impossible, at the animal level. Cell culture allows observation and manipulation of viral-host cell interactions at the cellular level. Cell culture experiments can provide powerful insights into the mechanisms of the infection process, the replication of the causative virus in the host, and how the host cell responds to a viral infection. Bovine coronavirus has been adapted to cell culture and can be studied at the cellular level. It produces a subtle but recognizable cytopathic effect and easily establishes persistent infections in vitro.

Preventing a virus infection is fundamentally more economical than treating or enduring one. Manipulation of an infectious agent so it can be used to prevent disease, perhaps by stimulating the host's immune system to resist infection, is the goal of vaccines. The genes of the four major structural proteins of BCV have been cloned and sequenced. This information can now be used to determine which structural proteins are involved in virus entry and which ones are important in stimulating the host's immune response. Information gained from experiments such as these can be directly applied to the construction of subunit or recombinant vaccines against BCV. Taken one step further, integration of specific BCV genes into the bovine germ cell line could possibly result in transgenic livestock that are resistant to BCV infection.

This study was undertaken to establish whether the HE gene of BCV could be stably integrated and subsequently expressed in a mammalian cell line. If accomplished, this would be an important step in analyzing virus-host interactions at the cellular level. Expressing the HE protein of BCV in such a system would theoretically allow one to characterize:

1. The synthesis, assembly, structure and function of the HE protein.
2. The identity of a viral receptor and whether or not it is a surface glycoprotein.
3. The usefulness of in vitro expressed viral proteins as vaccine immunogens.
4. The feasability of generating transgenic animals with this protein.

Understanding the pattern of synthesis of the HE protein within the cell and identifying the BCV receptor were the primary objectives for this study.

Chapter 2

LITERATURE REVIEW

Expression of Viral Proteins in Mammalian Cells

Recombinant DNA technology has provided techniques to manipulate individual genes from any organism once the sequence of that gene is known. At the molecular level, a gene can be transferred into a completely different host cell and be accepted as part of the cell's own genetic makeup. If the foreign gene is recognized by the host cell's replicative machinery, it may be transcribed and translated into a recognizable protein product. Expressing foreign proteins in various host cells is the goal of in vitro expression systems.

The human hepatitis B surface antigen and the influenza virus hemagglutinin protein were the first two viral proteins to be expressed in mammalian cells (Moriarty, 1981; Gething and Sambrook, 1981). These viral genes were inserted into a replication defective SV40 virus which contained the SV40 promoter sequence recognizable by the primate CV-1 host cell line. The recombinant DNA and a helper virus were transfected into the host cell and the resultant viral infection produced the foreign viral protein. The recombinant viral proteins were identical to authentic hepatitis B surface antigen and influenza hemagglutinin and were expressed on the surface membranes of the host cell.

The technology for expressing viral genes in mammalian cells has opened the door for the detailed study of the function of any virus as individual protein in a controlled in vivo environment. Prokaryotic cells as well as insect cells have been extensively used to express vertebrate genes and genes from viruses of vertebrates. However, these cells are often not capable of proper post-translational modifications of the vertebrate gene products (Sambrook et al., 1989). An expressed vertebrate protein may require disulfide bond formation, glycosylation, or phosphorylation for proper folding and biological activity. A mammalian host cell would, therefore, most likely produce a recombinant

mammalian protein identical to the authentic protein, and recognize signals within the expressed protein for its transport to the cell surface membrane for secretion (Cullen, 1987).

In vitro expression systems can be used to meet several experimental objectives (Sambrook and Gething, 1988; Sambrook et al., 1989):

To identify the gene sequence required to synthesize a functionally correct protein.

To produce large amounts of proteins that are usually made in small quantities.

To study the biosynthesis and intracellular transport of proteins during their expression in various cell types.

To mutate regions within the expressed gene and determine the result of structural changes on the function of the protein.

To identify regions within the expressed gene that are antigenic or immunologically important.

To identify DNA sequences involved in the control of gene expression.

Two types of in vitro systems are used to express viral proteins in mammalian cells, transient and stable (Rigby, 1983). Transient expression systems use infectious viruses to introduce foreign genes into a host cell while stable expression systems often require transfection and integration of recombinant DNA into the host cell's genome for expression.

Infectious viral vectors such as SV40, polyoma, vaccinia, adenovirus, and retrovirus, have been used to transiently express various recombinant proteins. Transient expression systems produce enough product for functional and immunological studies within 48 to 72 hours (Cullen, 1987). These vectors produce large copy numbers of the inserted gene per cell and consequently large amount of product, and are excellent for short term studies. However, if cell lysis is required to obtain the protein, experiments need to be repeated for continued analysis of the recombinant protein product. Several viral proteins

have been expressed using infectious viral vectors (Table 2.1)

Vaccinia has proven to be the most promising infectious vector. This poxvirus is capable of accepting 25,000 base pairs of insert and remain capable of replication (Mackett et al., 1984). It has been successfully used to express several viral proteins. An additional advantage of vaccinia recombinants is their application as vaccines. Intradermal injections of recombinant vaccinia constructs containing the influenza hemagglutinin protein have induced protective antibody responses in rabbits (Panicali et al., 1983) and hamsters (Smith et al., 1983). This same construct has also been used in cell culture to study cytopathic T-cell response to the hemagglutinin protein (Bennink, 1984).

Stable expression systems usually use noninfectious vectors for expression. However, some infectious retrovirus vectors have been used to stably transform cells since they have a required integration step in their replicative cycle. Vectors derived from the bovine papilloma virus also establish stably expressing cell lines yet the foreign gene remains as an episome in the host cell's cytoplasm (Rigby, 1983).

Commonly, vectors used for stable integration are double-stranded DNA plasmids that contain viral sequences to promote expression (ie., promoters) and antibiotic resistance genes for selection in both procaryotic and eucaryotic hosts. A variety of expression vectors have been constructed that employ several different promoter sequences and have been successful in expressing viral proteins (Table 2.2). Stably transformed cells may express lesser quantities of the recombinant protein, but they are useful in long term studies. Noninfectious vectors also express the recombinant protein without other viral proteins being present in the host cell.

Some vectors used for stable expression contain promoters are transcriptionally weak or inactive in cell culture until they are induced by specific compounds added to the growth media. The mouse mammary tumor virus promoter and the metallothionein promoter are induced by the addition of

TABLE 2.1 Infectious Viral Based Vectors Used to Transiently Express Viral Proteins in Mammalian Cells

Vector	Gene Expressed	Reference
SV40	human Hepatitis B virus surface Ag	Moriarty 1981
SV40	Influenza virus hemagglutinin	Gething and Sambrook 1981
SV40	Mouse Hepatitis Matrix Protein	Rottier 1987
Adenovirus	SV40 Large T antigen	Thummel and Solnick 1981
Adenovirus	polyoma virus tumor antigens	Mansour 1985
Adenovirus	Middle T Antigen	Davidson 1987
Retroviruses		
Harvey murine sarcomavirus	HSV thymidine kinase	Wei 1981
Moloney Murine Leukemia Virus	HSV thymidine kinase	Tabin 1982
Poxvirus		
Vaccinia	thymidine kinase of HSV	Panicali and Paoletti 1982
Vaccinia	Adenovirus polymerase	Stunnenburg 1988
Vaccinia	M gene of VSV	Li 1988
Vaccinia	influenza HA	Panicali 1983/Smith 1983
Vaccinia	bovine Herpes gI and gIII	van Drunen-Littel 1989
Vaccinia	Hepatitis B Surface Ag	Smith 1983
Vaccinia	Infectious Bronchitis Virus S Protein	Tomley 1987
Vaccinia	FIPV Spike Protein	Vennema 1990

TABLE 2.2 Inducible Promoters Used to Stably Express Viral Proteins in Mammalian Cells

Promoter	Gene Expressed	Reference
MMTV	HSV-2 gG-2	Su 1988
MMTV	HSV-1 gC-1	Friedman 1989
MMTV	polyoma middle T Ag	Raptis 1985
MMTV	v-ras	Huang 1981
MMTV	v-mos	Papkoff 1984
MMTV	v-src	Jakobovits 1984
MMTV	adenovirus genes ?	Klessig 1984
Metallothionein*	Hepatitis B Surface Ag	Hsuing 1984
heat shock*	FIPV S Protein	DeGroot 1989

*non-viral promoter

steroids or cadmium ions to the growth medium, respectively (Cullen, 1987). These vectors are advantageous when expressing genes whose products may be toxic to the host cell or may alter cell growth. Initial experiments using the MMTV promoter to express the lacZ gene of E. coli showed levels of expression in uninduced cells to be equivalent to background levels in mock-tranformed cells (Lee et al., 1981; Hall et al., 1983). This observation suggests that the uninduced MMTV promoter is relatively transcriptionally inactive. Viral proteins have been expressed in mammalian cells using vectors containing inducible promoters (Table 2.2).

Coronavirus Structure and Replication

Coronaviridae infect a wide variety of animal species and cause acute, subacute, subclinical and/or persistent infections in their hosts. Typically, coronaviruses infect young animals and cause gastrointestinal or respiratory disease (Sturman and Holmes, 1983). Some coronaviruses cause diseases involving the central nervous system and many cause multisystemic disease (Table 2.3). When viewed by electron microscopy, coronaviruses are medium sized viruses that are spherical or pleomorphic in shape. The virion surface is covered by a characteristic "fringe" of club-shaped projections that are found on all coronaviruses thus far isolated.

The coronavirion contains three and sometimes four major structural proteins. The nucleocapsid protein (N; 50 kD), matrix protein (M; 25 kD), and spike protein (S; 200 kD) are common to all coronaviruses while six species contain an additional surface glycoprotein, the hemagglutinin-esterase protein (HE; 140 kD; Figure 2.1). The nucleocapsid protein is a phosphorylated glycoprotein intimately associated with the genomic RNA. The other three glycoproteins are imbedded in the viral membrane that is derived from host cell (Holmes, 1990). Two of the surface glycoproteins, S and HE, are thought to be

TABLE 2.3
Coronaviruses and Their Related Diseases

Common Name (Designation)	Natural Host	Clinical Presentation
Avian infectious bronchitis (IBV)	Chicken	Tracheobronchitis, nephritis oviduct hypoplasia
Bovine Coronavirus (BCV)**	Bovine	Gastroenteritis, diarrhea
Canine Coronavirus (CCV)	Canine	Gastroenteritis, diarrhea
Feline Enteric Coronavirus (FCV)	Feline	Enteritis
Feline Infectious Peritonitis Virus (FIPV)	Feline	Meningoencephalitis, peritonitis pleuritis, pneumonia, vasculitis disseminated granulomas
Foal enteritis coronavirus (FECV)[a]	Equine	Diarrhea
Hemagglutinating encephalomyelitis virus (HEV)**	Pig	Encephalomyelitis, gastroenteritis
Human enteric coronavirus (HCEV)	Man	Gastroenteritis
Human respiratory coronavirus (HCV; OC43 and 299E)**	Man	Common cold
Mouse hepatitis virus (MHV-JHM)**	Murine	Hepatitis, encephalomyelitis enteritis, interstitial pneummonia
Murine Coronavirus (DVIM)** Sialodacryoadenitis virus (SDAV)	Rat	Sialodacryoadenitis, rhinotracheitis keratoconjunctivitis
Parrot coronavirus[b]	Parrot	Diarrhea
Pleural effusion disease virus (RbCV)	Rabbit	Pleuritis, endocarditis
Porcine Epidemic Diarrhea Virus (PEDV)	Pig	Enteritis, diarrhea
Rabbit enteric coronavirus (RbECV)	Rabbit	Enteritis
Rat Coronavirus (RCV)	Rat	Pneummonia, rhinotracheitis
Transmissible gastroenteritis virus (TGEV)	Pig	Gastroenteritis, diarrhea
Turkey Coronavirus (TCV)**	Turkey	Enteritis, "blue comb disease"

[a,b]Not yet classified members.
**Coronaviruses containing the Hemagglutinin-Esterase glycoprotein.
(Adapted from Sturman and Holmes, 1983; Wege, et al., 1982)

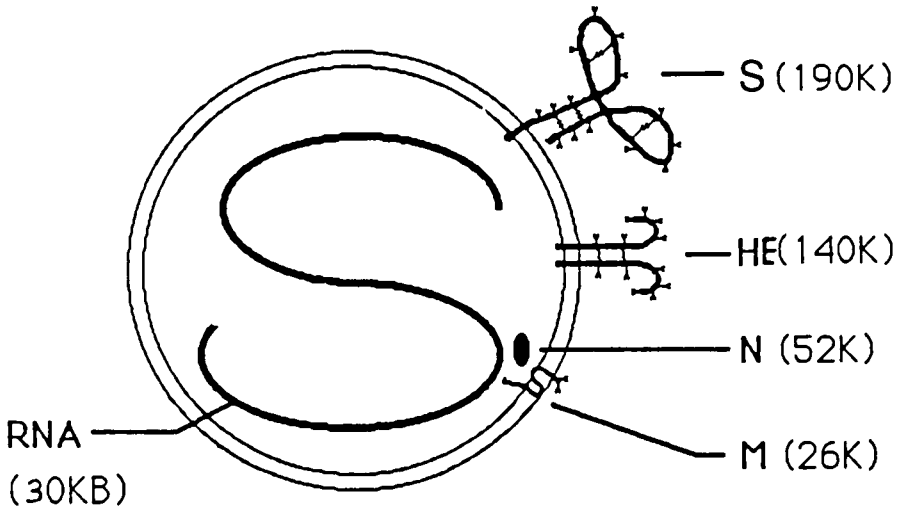


Figure 2.1 Schematic diagram of the bovine coronavirion.
The single stranded RNA genome is associated with the nucleocapsid protein (N). The lipid bilayer envelope contains three surface glycoproteins; the spike protein (S), the hemagglutinin-esterase protein (HE), and the matrix protein (M).

important in the attachment and fusion of the virion to the host cell.

Coronaviruses have the largest RNA genome yet characterized among the RNA viruses (27-30 kb; Fenner et al., 1987). The single stranded RNA genome is of positive polarity, capped and polyadenylated which allows it to serve as an infectious mRNA molecule. At the onset of infection, the viral RNA genome is released into the cell's cytoplasm where it serves as a template for the synthesis of a virus specific RNA-dependent RNA polymerase. This viral polymerase transcribes the viral genomic RNA to form a complementary full-length negative-sense RNA. This negative-sense RNA is used to transcribe subgenomic positive-sense mRNAs that encode other viral proteins and the positive-sense full length genome that is packaged into progeny virus (Lai et al., 1982).

The mRNAs of coronaviruses form a 3' nested set. The sequence of the shorter message is contained within the 3' end of the next larger message (Wege et al., 1981). Although only the 5' most end of each message is translated, coronavirus mRNAs, except for the shortest mRNA, contain sequence for more than one message. This phenomenon allows for the description of a 3' nested set of messages that are structurally polycistronic but are functionally monocistronic (Spaan et al., 1988; Figure 2.2).

Currently, it is thought that coronavirus mRNAs are synthesized by a discontinuous transcription process (Spaan, et al., 1983; Baric et al., 1983). The subgenomic mRNAs and the genome length RNA have been found to have an identical sequence at their 5' end designated as the leader sequence (Lai, et al, 1983, 1984; Spaan et al., 1983; 1984). Theoretically, the leader sequences are transcribed from the 3' end of the negative-sense template and dissociate as a complex with the polymerase (Holmes, 1990). The leader "fragments" and the polymerase bind to complementary intergenic regions of the negative sense template and form a transcriptional complex (Bredenbeek et al., 1987; Baric et al., 1987). Transcription proceeds from the 3' end of the leader and subgenomic

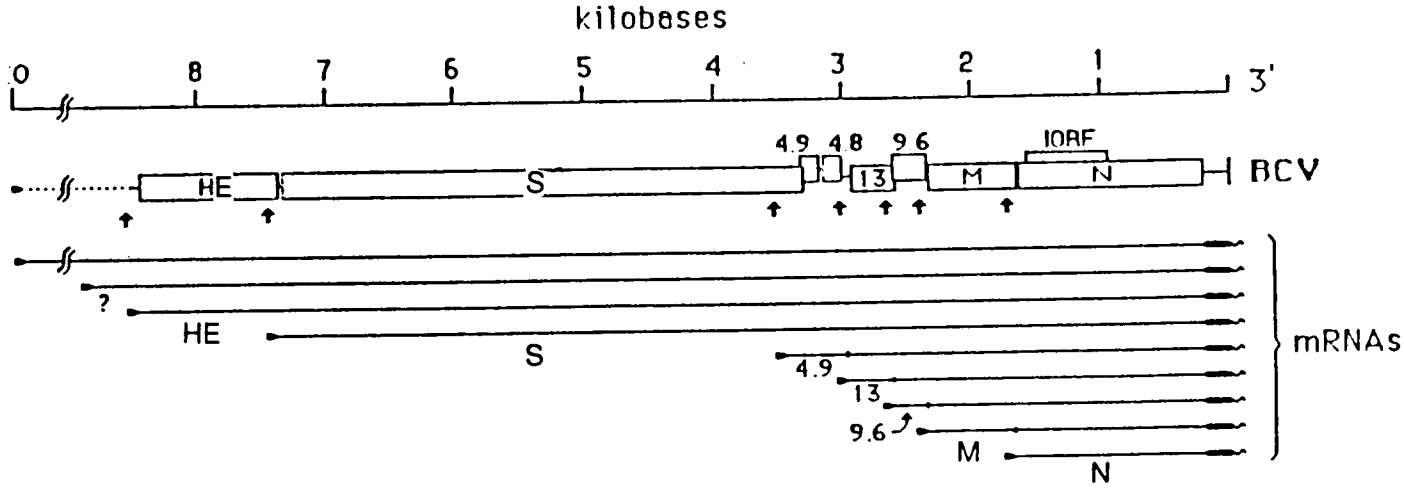


Figure 2.2 Gene order and arrangement on the bovine coronavirus genome illustrating the nested set arrangement of the BCV mRNAs, and the translation products of each mRNA. Open boxes represent genes and open reading frames. Vertical arrows indicate positions of the intergenic consensus sequence. Numbers indicate predicted molecular weight of putative nonstructural proteins; M, matrix protein; N, nucleocapsid protein; S, spike protein; HE, hemagglutinin-esterase protein; ? indicates unknown.

messages are made. Recent evidence shows that coronavirus mRNAs may undergo replication using the same replicational mechanism(s) as the genome (Sethna et al., 1989; Sawicki and Sawicki, 1990; Hofmann et al., 1990).

The mRNAs are translated in the host cell's rough endoplasmic reticulum and are processed in the smooth endoplasmic reticulum and Golgi where they undergo glycosylation and further modification. Virions are assembled as they bud into intracellular spaces of the endoplasmic reticulum and Golgi. The Golgi membranes give rise to vesicles which contain large numbers of infectious virus particles that exit the cell by exocytosis (Dubois-Dalcq et al., 1984; Holmes,1990; Figure 2.3).

In Vivo Expression of Coronavirus Proteins

The spike (S) protein of the murine hepatitis virus (MHV) has been expressed in insect cells using a baculovirus vector (Yoden et al., 1989). The recombinant protein was similiar in size to the authentic viral glycoprotein, however the recombinant form was not glycosylated. Serum from rats immunized with the recombinant S protein reacted in immunofluorescence and immunoprecipitation experiments with infectious MHV, however it failed to neutralize the infectious virus. This failure was suspected to be due to the lack of post-translational processing of the recombinant protein made in insect cells.

The matrix (M) protein of MHV has been expressed in mammalian cells using an SV40 based vector (Rottier et al., 1987). This envelope glycoprotein was found to accumulate in the perinuclear region of transfected cells. Expression of the M gene determined that the gene must carry a signal for this localization within the Golgi of transfected cells.

A recombinant vaccinia virus containing the spike protein (S) of the infectious bronchitis virus (IBV) showed immunogenic specificity with anti-IBV antibodies raised in rabbits by immunofluorescence and immunoprecipitation

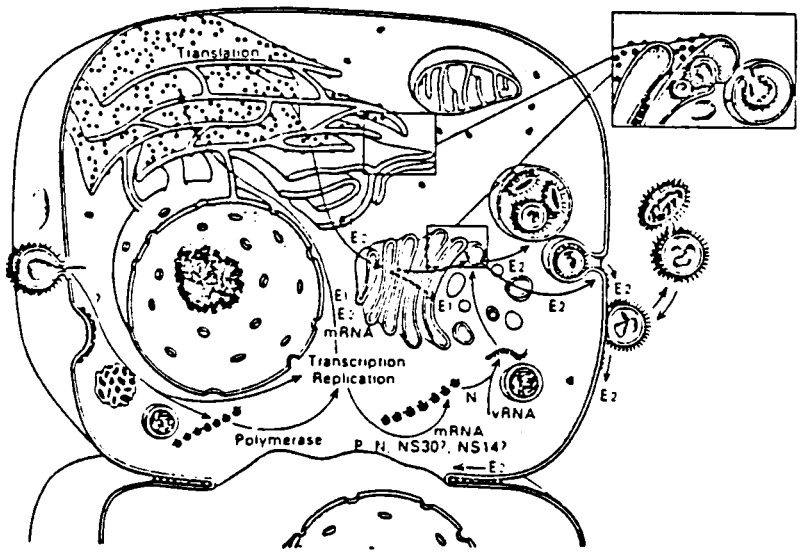


Figure 2.3 A model summarizing the events during replication, transcription, and assembly of a representative coronavirus, mouse hepatitis virus. E1, matrix protein; E2, spike protein; N, nucleocapsid protein; NS nonstructural proteins; vRNA, viral RNA (Taken from Dubois-Dalcq et al., 1984).

(Tomley, et al., 1987). This same construct produced antibodies against IBV in vaccinated mice which neutralized infectious IBV.

The S protein of the feline infectious peritonitis (FIPV) coronavirus was stably expressed in a bovine papilloma virus transformed mouse C127 cell line (DeGroot et al., 1989). The expression vector used contained the Drosophila heat-shock promoter. The transformed cells exhibited cell fusion when they were co-cultivated with FIPV permissive feline cells. The authors suggest that a FIPV receptor must be present for cell fusion to take place. Immunofluorescence data suggested that the majority of the recombinant protein remained in the cytoplasm, however, the authors propose that this "slow maturation" of the S protein may be required for efficient viral assembly.

A vaccina recombinant expressing the S protein of feline infectious peritonitis virus produced neutralizing antibodies in mice (Vennema et al., 1990). Subsequent vaccination of kittens failed to illict high titers of neutralizing antibodies. After challenging the vaccinated animals with infectious FIP, the vaccinated kittens succumbed earlier than did the control group immunized with vaccinia virus. The vaccine failure was considered to be due to an antibody-enhancement of infection. Interestingly, preliminary data from this same research group indicated that antibodies to the M and N proteins do not induce death after viral challenge.

The hemagglutinin-esterase protein of bovine coronavirus has been expressed in vivo using an infectious vaccinia virus expressing the T7 polymerase protein (Kienzle et al., 1990). The HE gene was subcloned into a plasmid under the control of the T7 polymerase promoter. The plasmid was transfected into vaccina virus-infected cells and the HE protein was expressed. The product formed disulfide linked dimers as found on the virion and was expressed on the host cell's surface membranes.

Chapter 3
MATERIALS AND METHODS

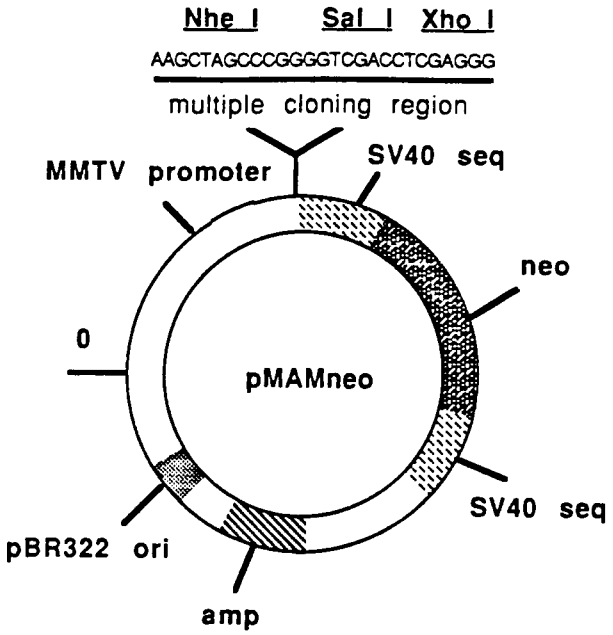
Subcloning the Hemagglutinin-Esterase Gene of the Bovine Coronavirus into a Mammalian Expression Vector

Mammalian Expression Vector: pMAMneo

A pBR322 based plasmid, pMAMneo (Clonetech Laboratories, Inc., Palo Alto, CA), was used to express the hemagglutinin-esterase gene of the bovine coronavirus (Figure 3.1). The plasmid (8.3 kb) contained an ampicillin resistance (amp) gene and a neomycin resistance (neo) gene. The amp gene allowed selection and amplification of recombinants in Esherichia coli (E. coli). The neo gene allowed selection of successfully transformed mammalian cells in culture medium containing aminoglycoside antibiotics, such as kanamycin, neomycin, and Geneticin^{R} (Sigma Inc., St. Louis, MO), also known as Antibiotic G418.

Cloning the HE gene into the pMAMneo vector places it under control of the mouse mammary tumor virus (MMTV) promoter. Adding dexamethasone (Sigma) to the culture medium (1×10^{-6} M) induces the MMTV promoter thus increasing production of the desired gene product three to five times over noninduced levels (Lee, 1981; Sardet, 1989). Proteins expressed under control of the MMTV promoter have been detected as early as five hours after induction and found to be increased to a maximum level at twenty two hours post-induction as determined by immunoprecipitation (Su and Courtney, 1988).

pMAMneo plasmid DNA (2.5 μg; Lot M201), was linearized using the restriction endonuclease Sal I (New England Biolabs, Beverly, MA; NEB) and blunt-ended in the presence of Klenow enzyme (NEB) and dNTPS (Sigma) as described (Current Protocols in Molecular Biology, 1987). Dephosphorylation



amp = ampicillin resistance gene
neo = neomycin resistance gene
MMTV = mouse mammary tumor virus promoter

Figure 3.1. Map of the mammalian expression vector pMAMneo (Clonetech, Palo Alto, CA). The ampicillin resistance gene (amp) allows selection and amplification of recombinants in E. coli. The neomycin gene (neo) allows selection of successfully transfected mammalian cells in the presence of Antibiotic G418. The mouse mammary tumor virus (MMTV) promoter is induced by the addition of dexamethasone to the culture medium.

was achieved by adding alkaline phosphatase (Boehringer Mannaheim Biochemicals, Indianapolis, IN) as described (Manniatis et al., 1982).

Bovine Coronavirus Hemagglutinin-Esterase Gene

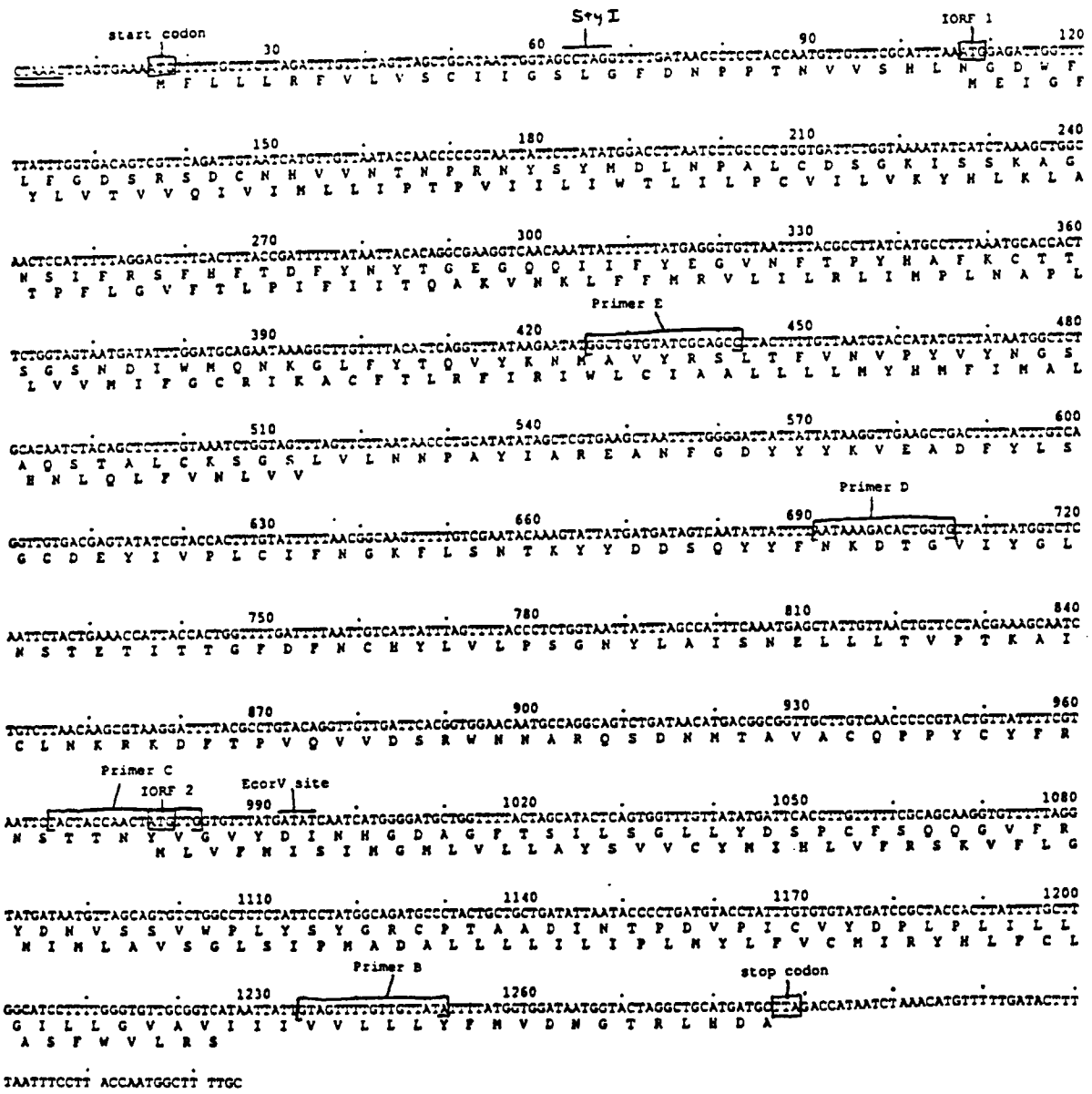
The BCV HE gene was obtained as a cloned, double-stranded DNA fragment that could be removed from the pGEM-3Z plasmid (T. Kienzle, Ph.D. dissertation, 1989; Promega, Madison, WI) using EcoR I and Hind III restriction enzyme digests (NEB). The purified fragment was blunt-ended in the presence of Klenow enzyme and DNTPs and subcloned into the blunt-ended Sal I site of the multiple cloning region of the pMAMneo plasmid. The total HE gene fragment consisted of 1341 basepairs and included the HE gene (1272 basepairs), a sequence of 15 basepairs from a region just upstream of the gene's start codon, and a sequence of 54 basepairs following the gene's stop codon (Fig. 3.2).

Construction of Plasmids Containing the Bovine Coronavirus Hemagglutinin-Esterase Gene

Preparations of purified linearized vector and insert were separately electrophoresed on a 1% agarose gel (Sea-Kem^R, GTG; FMC Bioproducts, Rockland, ME) in the presence of 0.5 μg/ml ethidium bromide (EtBr; Sigma) to determine relative intensity and sharpness of DNA bands. Band intensity was used to estimate the quantity of DNA to be used in subsequent ligations.

Ligation reactions were designed to contain molar ratios of insert to vector DNA of 4:1 and 3:1. These ratios were chosen to increase the probability of proper reaction products (Manniatis et al., 1982). Ligations were catalyzed by T4 DNA ligase (NEB) and T4 RNA ligase (NEB) under the appropriate buffer conditions (Current Protocols in Molecular Biology, 1987). T4 RNA ligase

Figure 3.2 The Sequence of the Gene Fragment Used for Expression of the BCV Hemagglutinin-Esterase Protein in Mammalian Cells. The start codon of the HE gene and the stop codon are indicated. The Sty I and EcoRV site used to make the probe used for colony hybridization are shown. Internal oligonucleotide primers prepared by T. Kienzle and capable of binding to positive-sense (genome) sequence are indicated. Oligo E was used in PCR reactions in ths study.


Sty I
start codon
IORF 1
Primer E
Primer D
Primer C
IORF 2
EcorV site
Primer B
stop codon

increases efficiency of blunt-end ligations by a yet unknown mechanism (Sugino, 1977).

Following an overnight incubation at room temperature, aliquots of each reaction were electrophoresed on a 1% agarose gel containing EtBr to confirm that ligation had taken place.

Transformation and Identification of Recombinants

Varying amounts of each ligation reaction were transformed into previously prepared E. coli cells (JM105, JM89 or HB101) made competent by a modified Hanahan procedure (Appendix). Aliquots of each transformation reaction were plated on selective YT medium plates containing, 1.5% agarose (Difco Laboratories, Detroit, MI) 50 μg ampicillin/ml (Sigma; YT-amp plate). Plates were incubated at 37°C overnight and ampicillin resistant colonies were selected and screened for the presence of HE gene sequence by colony hybridization (Manniatis, 1972). Positive and negative controls were transformed E. coli containing pGEM-3Z-1-HE (gift from T. Kienzle) and pMAMneo, respectively. Individual colonies were transfered to a YT-amp plate in a numbered grid pattern and incubated overnight at 37°C. The transferred colonies were adsorbed onto nitrocellulose filters (S&S NC^R BA85; Schleicher and Schuell, Keene, NH), denatured and prehybridized as described (Appendix).

A probe was made from a 930 base pair (bp) fragment of the HE gene isolated from an EcoR V and Sty I (NEB) enzyme digest of the pGEM4Z-5, a plasmid containing the HE gene (T. Kienzle, Ph.D. dissertation, 1989; Figure 3.2). The fragment of DNA was isolated from an agarose gel by electroelution and nick-translated in the presence of γ^{32}P-dCTP (ICN Radiobiochemicals, Inc., Costa Mesa, CA) as described (Current Protocols in Molecular Biology, 1987). The lableled probe had a specific activity of 2.0×10^7 cpm/min.

The treated filters and probe (1.36×10^5 cpm/cm^2) were incubated overnight

in a 55°C waterbath and washed twice with 2x SSC, 0.5% SDS at room temperature and then with 0.1x SSC, 0.5% SDS at 55°C for 30 minutes (1x SSC contains 0.15 M NaCl, 0.015 M trisodium citrate [pH 7.0]). Autoradiographs of the washed filters allowed visualization of colonies containing the HE gene sequence. Positive colonies were grown in liquid cultures and DNA purified using the alkaline lysis method and precipitated in 4 M NaCl and 12% polyethylene glycol (Sigma) as described (Promega Technical Bulletin 009).

Orientation of the HE gene insert was determined by two methods: sequencing and restriction enzyme digests. Sequencing by the dideoxynucleotide chain termination method was performed using the Sequenase Kit (Version 2.0; US Biochemical Corp., Cleveland, OH) and $\alpha^{32}P$ labeled dATP (600 Ci/mmol; ICN). The 5' and 3' ends of the HE gene insert were determined by using primers corresponding to the unique regions of the pMAMneo vector. These were synthesized in the UT Biology Facility(Figure. 3.3). Restriction endonucleases, EcoR V and Nhe I or EcoR V and Xho I (NEB), were used to digest plasmid DNA containing the HE gene and the resulting fragments were electrophoresed on 1% agarose gels containing 0.5 μg/ml EtBr and visualized by UV illumination.

Plasmid preparations of pMAMneo vector, pMAMneo-HE sense and pMAMneo-HE antisense were made as described above. Concentrations of DNA were determined by observing the absorbance values of 1:500 or 1:1000 dilutions of DNA in a spectrophotometer (Bausch-Lomb; Spectronic 1001). Absorbance values at two wavelengths (A_{260} and A_{280}) were used to determine the quantity and purity of each preparation.

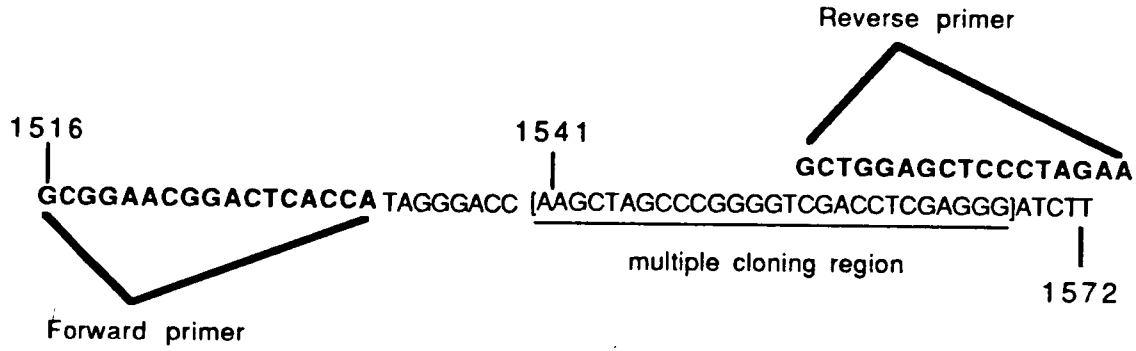


Figure 3.3. Oligonucleotide primers used for sequencing and for the polymerase chain reactions on inserts contained within the pMAMneo vector. Numbers shown refer to base positions within the pMAMneo vector.

Establishment of Stably Transformed Mammalian Cells Containing the Hemagglutinin-Esterase Gene

Mammalian Cells: Human HRT-18 and Murine L929

Human rectal carcinoma cells (HRT-18; Tompkins et al., 1984) and murine L929 cells (American Type Tissue Culture) were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco Laboratories, Grand Island, NY) containing 50 μg/ml gentamicin (Sigma) and supplemented with 10% fetal calf serum (FCS; Hyclone, Logan UT or Sigma) as previously described (King and Brian, 1982; Hogue et al., 1984). Human rectal tumor cells infected with BCV were used as positive controls for evaluation of HE protein expression.

Infectious Bovine Coronavirus

Previously prepared stocks of infectious bovine coronavirus (Mebus Strain) were prepared and maintained at $^{-}80^{o}C$. Virus was propagated and harvested from HRT-18 cells as previously described (King and Brian, 1982; Hogue, 1984) Virus titers were assayed by plaque formation under 1% agarose-DMEM using monolayers of HRT-18 cells as described (Guy and Brian, 1979).

Transfection of Mammalian Cells Using Lipofectin Reagent

HRT-18 and L929 cells were cultured in 150 cm^2 polystyrene flasks (Corning, Corning, NY) containing DMEM, 10% FCS(Sigma) and gentamicin (Sigma; 50 μg/ml). Flasks were maintained in a humidified incubator at 37°C, under 8% CO_2.

Cells used for transfections were passaged by trypsinization using recrystallized trypsin (Calbiochem) and trypsin diluent containing EDTA.

Trypsinized cells were resuspended and diluted 1:10 in growth medium and reseeded in 25 cm^2 polystyrene flasks (Corning). Passaged cells were allowed to grow to approximately 80% confluency prior to transfection.

Transfections were performed using 5-10 μg of DNA from plasmid preparations of pMAMneo-HE sense, pMAMneo-HE antisense, and pMAMneo vector. The DNA was mixed with 30 -50 μg of Lipofection™ Reagent (Bethesda Research Laboratories, Inc., Gaithersburg, MD) according to described protocols (Appendix). Flasks were incubated at 37°C for 24 hours, refed with growth medium and incubated for an additional 48 hours prior to passaging and adding selection medium.

Passaging and Selection of Transformed Cells

Transfected cells were trypsinized and reseeded into 24 or 96 well plates (Corning) at dilutions providing 10 to 20 cells per well. Remaining cells were seeded into 25 cm^2 flasks and retained as nonclonally selected cell populations. After 14 to 21 days, clonally selected cells were trypsinized and seeded into 25 cm^2 flasks, 35x10 mm Petri dishes (Corning) and multi-chambered plastic slides (LabTek; Nunc, Inc., Napiersville, IL). Colonies of cloned cells were numbered consecutively (ie., 1, 2, 3...) and grown to confluency prior to induction and evaluation of HE protein expression. Flasks of nonclonally selected cells were designated alphabetically (ie., A, B, C...) and maintained as stock populations.

Culture medium used to passage the cells from the original transfection flask contained the selection agent, Antibiotic G418. Previous work describing the selection of mouse L cells transfected with the pMAMneo plasmid used 400 μg/ml of G418 in the culture medium (Sardet, 1989). Therefore, transfected mouse fibroblasts (L929) used in these experiments were selected with this same level of G418. Various levels of G418 (200 μg/ml to 1200 μg/ml) were evaluated to

determine the optimum concentration of antibiotic to be used in selecting transfected HRT-18 cells.

Screening and Confirmation of HE Gene Integration by the Polymerase Chain Reaction

Transformed HRT-18 and L929 cells were screened for the presence of the HE gene using the polymerase chain reaction. The PCR requires a nucleic acid template, two primers capable of annealing to the template and extending it in opposite directions, and proper reaction conditions containing specific concentrations of dideoxynucleic acid triphosphates (dNTPs), magnesium ions and Taq I polymerase (Perkin-Elmer Cetus, Corp., Norwalk, CT or Promega). The Taq I polymerase is a bacterial enzyme that is active at a higher temperature than other polymerases and is well suited to the specific reaction conditions of PCR.

In these experiments, PCR reactions were performed on DNA isolated from aliquots of trypsinized transformed cells (Smithies, 1988). Cells were diluted in PBS and pipetted into siliconized snap cap tubes (Robbins Scientific, Sunnyvale, CA), centrifuged (Savant, 7000 rpm, 5 min) and the supernatant was removed by aspiration. The cell pellet was treated with 1 μg/μl Proteinase K (Sigma) and diluted into PCR buffer containing dNTPs, primers and Taq I, as described (Appendix). Reaction products were analyzed on a 1% agarose gel containing EtBr and viewed by UV illumination.

Two different pairs of primers were initially used to amplify a 600 basepair (bp) fragment or a 1400 bp fragment from the DNA template. The 600 bp fragment was generated using a HE specific primer (melting temperature = 54°C; 3'CCG-ACA-CAT-AGC-GTC-CC5'; Oligo "E", as designated in T. Kienzle, Ph.D. dissertation, 1989) and the forward vector primer (melting temperature = 56°C;

5'GCG-GAA-CGG-ACT-CAC-CA3'). The generated 600 bp fragment identified the HE gene specificity and orientation in the expression vector. The 1400 bp fragment was the product of the forward vector primer and the vector reverse primer (melting temperature = 54°C; 3'GCT-GGA-GCT-CCC-TAG-AA5') that amplified the entire HE gene within the pMAM-neo vector's multiple cloning region. The size of the amplified fragments were estimated by visual comparison to corresponding fragments of a 1 KB Ladder Marker (NEB) on a 1% agarose gel.

Cells found to contain the entire HE gene sequence by PCR were maintained as transformed cell lines. They were routinely monitered by PCR to ensure the continued integration and size status of the HE gene sequence on subsequent passages.

Southern Blot Analysis

Agarose gels used to visualize the PCR-generated DNA fragments were used for Southern Blot analysis. The DNA fragments were denatured in the gel with alkali, neutralized with Tris-HCl [pH=7.4] and vacuum-blotted onto 10 cm x 14 cm nylon membranes (Nytran)(Schleicher and Schuell) using a 2016 Vacugene (LKB Products, Bromma, Sweden). The DNA was covalently bound to by exposing the dry filter to 286 nm UV illumination for 2 minutes. The filter was hybridized and probed with an oligonucleotide (Oligo E) end-labeled with γ^{32}P-CTP (ICN; 3.7 x 10^7 cpm/min) as described (Current Protocols in Molecular Biology, 1987). The probe was specific for HE gene sequence and would anneal only to DNA generated by the PCR reaction.

The probed nylon membranes (2.6 x 10^5 cpm/cm^2) were washed as described above for colony hybridization and autoradiographed or analyzed on the Ambis Radioanalytic Imaging System™ (Ambis).

Detection of the Hemagglutinin-Esterase Protein in Cells Containing the Integrated Hemagglutinin-Esterase Gene

Induction of the Mouse Mammary Tumor Virus Promoter

The MMTV promoter was induced by supplementing the culture medium with $1x10^{-6}$ M dexamethasone (9-α-fluoro,16-α-methyl-prednisolone; Sigma). Transfected cells were induced and evaluated 24 hours post-induction to detect the expression of the HE protein by hemadsorption, indirect immuno-fluorescence, and immunoprecipitation.

Hemadsorption of Murine Erythrocytes

Nontransformed HRT-18 or L929 cells were grown in 35x10 mm Petri dishes until they were 80% confluent and infected with BCV with approximately 5 plaque forming units per cell. Infected cells served as positive controls and uninfected HRT-18 or L929 cells served as negative controls for hemadsorption assays. Transfected cells were induced with dexamethazone 24 hours prior to analysis. Medium was aspirated from the dishes and the cells were washed with Earle's Balanced Salt Solution (EBSS; KC Biologics, Kansas City, KS) containing 100mM HEPES (Sigma) as a buffer.

A suspension of murine erythrocytes, collected in Alsevier's anticoagulant solution (Gibco) and diluted in phosphate buffered saline (PBS), was pipetted onto the HRT-18 or L929 cells and the dishes were incubated at room temperature for 1 hour. The erythrocytes were aspirated and the cells were gently washed four times with HEPES-buffered EBSS and viewed under a light microscope.

Indirect Immunofluorescence

Transfected cells were grown on multi-chambered plastic slides (Lab-Tek; Nunc) until they were 60-80% confluent. Infected HRT-18 served as positive controls while uninfected HRT-18 or L929 cells served as negative controls. Transfected and control cells were induced with dexamethazone 24 hours prior to fixation. Medium was removed by aspiration and cells were washed three times with buffered EBSS. They were prepared for surface or internal indirect immunofluorescence by treatment with 4.0 % paraformaldehyde (Sigma) for 15 minutes at room temperature or 100% ethanol at -20°C, respectively.

A polyclonal antibody (Rabbit D; Hogue et al., 1984) previously made in our laboratory against the HE (gp65) of BCV was used as the primary antibody to detect expression of the HE protein. This polyclonal antibody was made by subcutaneously injecting a rabbit with BCV HE protein isolated from a polyacrylamide gel. Rabbit α-HE Rabbit D antibody was diluted 1:80 in HEPES-buffered EBSS, applied to the fixed cells and incubated at 37°C for 1 hour. Excess antibody was removed by washing the cells with HEPES-buffered EBSS.

The second antibody used to visualize the expression of the HE protein was commercially available FITC-conjugated goat anti-rabbit IgG (BMB; Lot 21013) diluted 1:50 in HEPES-buffered EBSS, applied to the fixed cells and incubated at 37°C for 1 hour. The slides were rinsed with HEPES buffered EBSS, covered with glass coverslips and viewed under UV illumination using a Nikon fluorphot microscope and a Nikon high pressure mercury light source.

Monoclonal antibodies were also used to evaluate the expression of the HE protein. They were from murine ascites fluid and classified as G_2 isotypes (A gift from M. Parker; Veterinary Infectious Disease Organization, Saskatoon, Canada) and used as described above with the exception that FITC-conjugated goat anti-mouse IgG (BMB; Lot 24049) was used as the second antibody. Three

different monoclonal antibodies designated HC10-5, 56-40, and BD9-8C were used. Hereafter, for simplicity, these are designated mAb1, mAb2 and mAb3, respectively.

Immunoprecipitation

Transfected HRT-18 cells or L929 cells were grown in 35x10 mm tissue culture dishes, induced as described above and labeled with ^{35}S-TransLabel (ICN; Appendix). Following induction and the addition of label, cells were incubated 24 hours in DMEM lacking methionine to allow incorporation of the labeled amino acids into newly synthesized cellular proteins. Cells were harvested and cell lysates were mixed with the polyclonal antiserum against the HE or one of the three different monoclonal antiserums following the protocol of Anderson and Blobel, 1983).

Control cell lysates (50 μl) and transfected cell lysates (50 to 250 μl) were incubated with 5 μl of undiluted polyclonal or monoclonal antibody overnight at 4°C. Protein-antibody complexes were precipitated and adsorbed to Protein Sepharose beads (Pharmacia). The beads were washed five times to remove unbound cellular proteins, the immunoprecipitated proteins were solubilized in Laemmli Standard treatment buffer and electrophoresed on an 10% polyacrylamide gel with or without beta-mercaptoethanol following the protocol of Laemmli (Laemmli, 1970).

Commercially prepared ^{14}C-labeled protein markers (Rainbow Markers; range 14,300-200,000 daltons: lysozyme 14.3 kD; carbonic anhydrase 30.0 kD; ovalalbumin 46.0 kD; bovine serum albumin 69.0 kD; phosphorylase a 92.5 kD; and myosin 200.0 kD; Amersham Inc., Arlington Heights, IL), ^{14}C-labeled ovalbumin (Amersham) and immunoprecipitated HE protein from infected HRT cells were used as known protein size markers. The resultant gels were dried and radiolabeled protein bands were observed with the Ambis System. Autoradiographs were made as permanent images of the immunoprecipitation gels.

Chapter 4
RESULTS

Isolation of Recombinants Containing the Hemagglutinin-Esterase Gene in Both Orientations

Ligated constructs were transformed into competent E. coli cells as described. One transformation using 10-20 ng supercoiled DNA/ 4.7 x 10^7 cells resulted in 313 colonies. To identify which transformants contained the HE gene, colonies were screened by colony hybridization using a labeled probe specific to the HE gene. This procedure yielded eight colonies that were positive (Figure 4.1).

Positive colonies were amplified and the resulting DNA was isolated and analyzed by restriction enzyme digests. One clone, designated HE6, was determined to be in the sense orientation and one clone, designated HE8, was determined to be in the antisense orientation. Linearization of these two clones by restriction enzyme digestion confirmed that only one copy of the HE gene sequence had been subcloned into the pMAMneo vector. Six other colonies that were positive by colony hybridization gave incomplete digests or improper band sizes following restriction enzyme digestion.

The two chosen clones, HE6 and HE8, were sequenced at the 5' vector-insert junction. Sequencing confirmed orientation of the HE gene and the actual number of bases between the 3' end of the MMTV promoter and the start codon of the HE gene sense construct, HE6. Translating the bases by computer in all three reading frames showed that no additional start or stop codons prior to the start of the HE gene had been created as a result of subcloning (Figure 4.2).

Figure 4.1 Colony hybridization of bacterial colonies transformed with pMAMneo containing the HE gene. Bacterial colonies were adsorbed onto nitrocellulose filters and probed with a ^{32}P-labeled HE-specific oligonucleotide (Oligo E; Kienzle, Ph.D. dissertation, 1989). Positive control colonies containing the HE gene sequence in pGEM3Z are indicated by + signs. Colonies determined to contain the HE gene insert in the sense (6) and antisense (8) orientation are numbered.

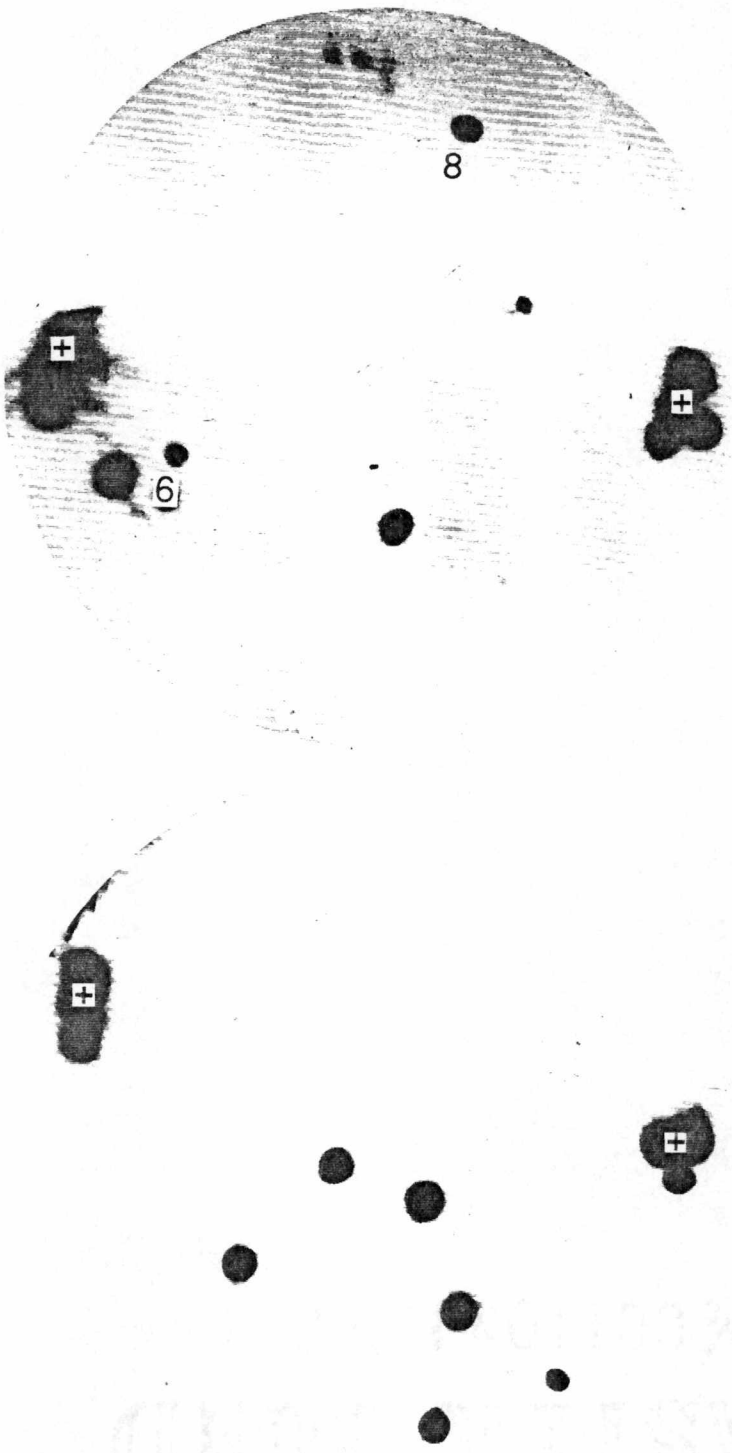
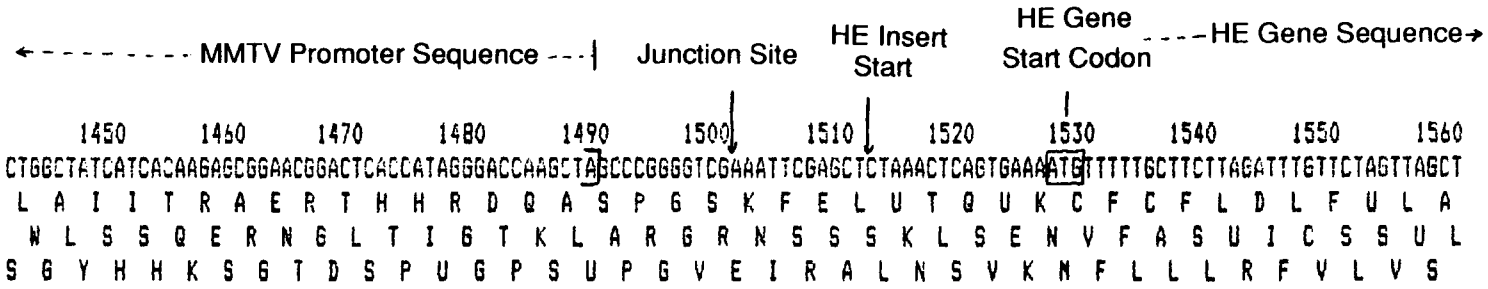

8
6

Figure 4.2 Translational analysis of sequence at the junction of the 3' end of the mouse mammary tumor virus promoter and the 5' end of the HE gene insert. The analysis determined that there were no additional start or stop codons created while subcloning the HE gene into the expression vector. Translational analysis was performed by Microgenie software (Queen and Korn, 1988).


MMTV Promoter Sequence
Junction Site
HE Insert Start
HE Gene Start Codon
HE Gene Sequence
1450 1460 1470 1480 1490 1500 1510 1520 1530 1540 1550 1560
CTGGCTATCATCACAAGAGCGGAACGGACTCACCATAGGGACCAAGCTAGCCCGGGGTCGAAATTCGAGCTCTAAACTCAGTGAAAATGTTTTTGCTTCTTAGATTTGTTCTAGTTAGCT
L A I I T R A E R T H H R D Q A S P G S K F E L U T Q U K C F C F L D L F U L A
W L S S Q E R N G L T I G T K L A R G R N S S S K L S E N V F A S U I C S S U L
S G Y H H K S G T D S P U G P S U P G V E I R A L N S V K N F L L L R F V L V S

Transfection of Mammalian Cells With pMAMneo Vector Containing the HE Gene Sequence in Both Orientations

Human rectal tumor (HRT-18) and mouse fibroblasts (L929) cells were transfected with pMAMneo vector, HE6 and HE8 DNA using Lipofectin™ Reagent as described above. Transfected cells were refed with growth medium 24 hours following the addition of Lipofectin thus allowing the cells to recover quickly and to grow to confluency within 72 of transfection.

Successfully transformed cells were selected in 14 to 21 days in the presence of 600 μg/ml of G418. Concentrations of G418 above this level did not shorten the selection time for transformed HRT-18 cells. Selection of transformed L929 cells occurred in 10 to 14 days using G418 at concentrations of 400 μg/ml. Transformed HRT-18 and L929 cells were maintained in growth medium containing 400 μg/ml of G418 during expansion, induction and analysis for HE gene expression.

For the first transfection, 10 μg each of supercoiled DNA from midipreps of pMAMneo, HE6 (sense) and HE8 (antisense) were transfected into HRT-18 cells. Populations of transformants were established on the basis of G418 resistance. These nonclonally selected cells were evaluated for expression of the HE protein by indirect immunofluorescence and hemadsorption.

For the second transfection, 10 μg each of linearized DNA from midipreps of pMAMneo, HE6 (sense) and HE8 (antisense) were transfected into HRT-18 cells. The plasmid DNA was linearized at the 3' end of the HE gene using the restriction enzyme Xho I (NEB; Figure 3.1). The DNA was linearized in an effort to increase the frequency of integration (Bill Lapps, personal communication).

Transformants were clonally selected as described and screened by PCR. Linearization of the transformed DNA did not noticeably affect transfection efficiency nor the frequency of HE gene integration as judged by the number of

clones selected and the results of PCR analysis on the transformants obtained from the second and third transfections.

For the third transfection, 15 μg of supercoiled midiprep DNA of HE6 (the sense orientation of the HE gene) was transfected into HRT-18 and L929 cells. Successfully transfected cells were clonally selected as described above. Integration of the HE gene was routinely monitored by PCR analysis prior to evaluation of HE expression by immunoprecipitation and indirect immunofluorescence.

Passaging and Establishment of G418 Resistant Cell Lines

Transformed HRT-18 cells from the first and second transfections (pMAMneo, HE sense and HE antisense) were passaged for over a period of three months (approximately 6 to 8 passages). Two HE sense cell lines (Clone 8 and Clone 9) and several antisense HE gene and pMAMneo cell lines were established.

Transformation of HRT-18 cells adversely affected their growth rate and viability. During the selection process cells often assumed strange shapes and were not viable after trypsinization. This fragility made it very difficult to grow populations of cells and to evaluate them by any of the described procedures. Increasing the serum concentration in the growth medium to 15% did not aid cell survival rates.

Transformed HRT-18 and L929 cells from the third transfection produced one HRT-18 clone and seven L929 clones containing the HE gene in the sense orientation as determined by PCR. The HRT-18 clone was negative for the HE gene by PCR and was not further evaluated. The seven L929 clones (Clones 1, 2, 3, 4, 6, 7, 11) were evaluated for expression of the HE protein.

In general, transformed HRT-18 cells containing the pMAMneo plasmid grew slower than untransformed HRT-18 cells. However, HRT-18 cells transformed by the HE sense and antisense constructs grew at an even slower rates. Clonal selection of HRT-18 cells was additionally difficult due to clumping of the cells

during trypsinization and eventual cell death. Transformed L929 cells grew slower than nontransformed L929 cells, but they grew faster and were easier to passage than transformed HRT-18s.

Occasionally, cells with an abnormally large and "flattened" appearance as well as multinucleated cells were observed among the transformed HRT-18 cells. Some L929 clones contained cytoplasmic vacuoles following induction but the other aberrant forms were not formed. Transformed L929 cells containing the HE sense construct grew slower and appeared more rounded when confluent than did nontransformed L929 cells (Figures 4.3, 4.4, and 4.5).

Evaluation of Expression of the Hemagglutinin-Esterase Gene

Polymerase Chain Reaction

Polymerase chain reactions (PCR) were used to screen transformants from the second and third transfections for the presence of the HE gene. These results are summarized in Table 4.1. Two HRT-18 HE sense clones (Clone 8 and Clone 9) were positive by PCR for the HE gene. Clone 9 was used for Southern blot analysis and Clone 8 was used for immunoprecipitation experiments (Figure 4.6). Selected HRT-18 cells from the third transfection yielded one HRT-18 clone that was negative for the HE gene as determined by PCR.

Screening L929 transformants from the third transfection by PCR yielded seven clones (Clones 1, 2, 3, 4, 6, 7, and 11) that were positive for the HE gene sense orientation. Repeated PCR analysis of clonally selected L929 cells revealed that three clones (Clone 1, 3 and 4) became negative for presence of the HE gene sequence within two consecutive passages (Figure 4.7). Clones 2, 6, 7, and 11 remained positive for the HE gene throughout five PCR analyses that continued for a period of three months at which time these analyses were ended and the cells

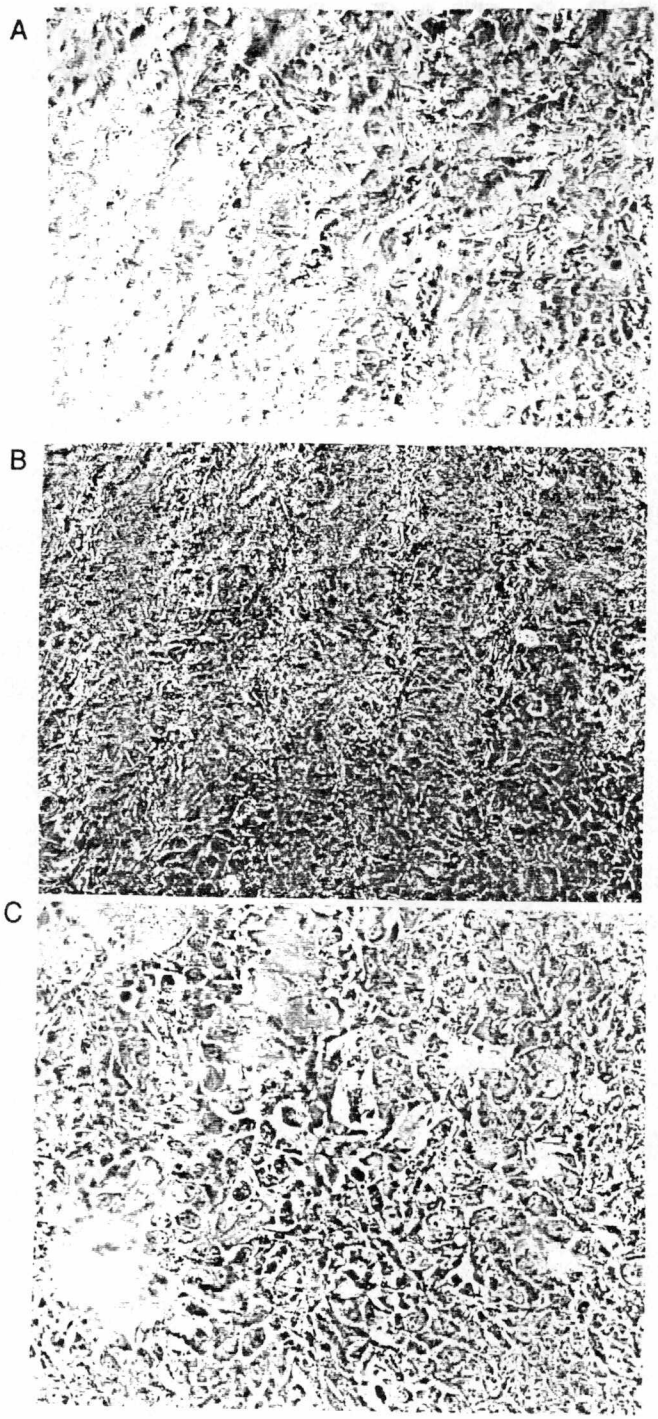


Figure 4.3 Light micrographs of confluent HRT-18 cells transfected with pMAMneo (A); pMAMneo/HE6 (B); and pMAMneoHE8 (C).

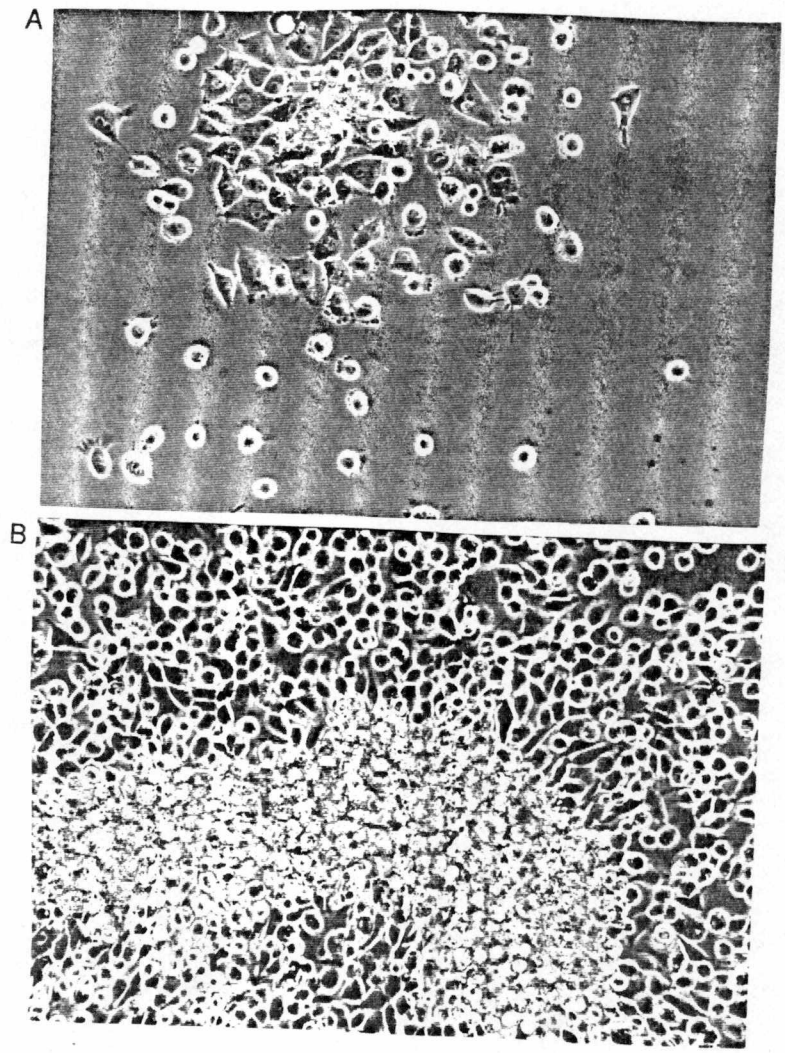
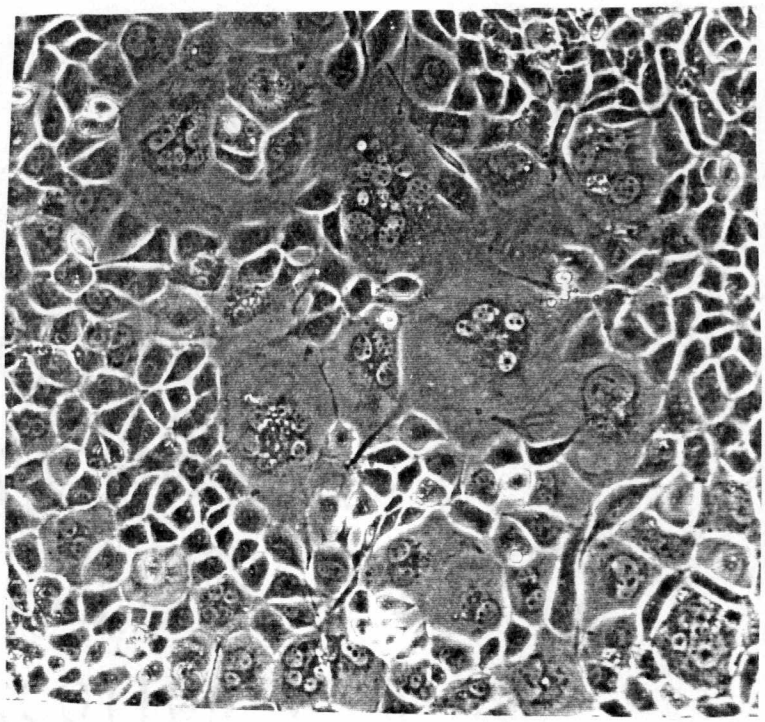


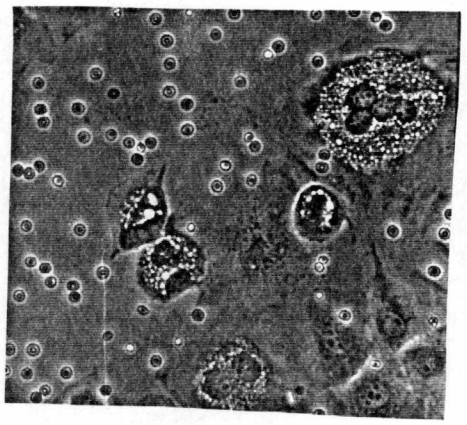
Figure 4.4 Light micrographs of murine L929 cells transfected with pMAMneo-HE sense following selection with 400 μg/ml of Antibiotic G418. The cells are shown shortly after selection as an established colony (A); and as an established island of cells with areas of rounded closely packed cells (B). As clones of cells reached the density as shown in (B), they were trypsinized and passaged into flasks for clonal expansion into established cell lines.

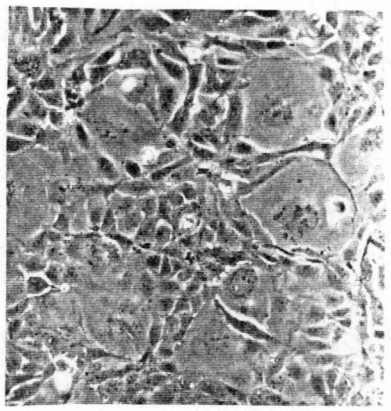
Figure 4.5 Light micrographs of transformed L929 cells and HRT-18 cells exhibiting enlarged irregular shapes andcytoplasmic vacuoles. (A) Noninduced pMAMneo-HE sense transformed L929 cells, (B) Induced pMAMneo-HE sense tranformed L929 cells, and (C) Noninduced pMAMneo-HE sense transformed HRT-18 cells.

A



B





C

TABLE 4.1 Polymerase Chain Reaction Results on HE Gene Transformed L929 Cells

Clone Number	Date of Polymerase Chain Reaction			
	8/16/90	9/13/90	9/24/90	10/25/90
1	+	-	dead	
2*	+	+	+	+
3	+	-	-	-
4	+	-	dead	
6**	+	+	+	+
7	+	+	+	+
11*	+	+	+	+

*Weakly positive for fluorescence on 9/24/90.

**Positive for fluorescence on 9/24/90.

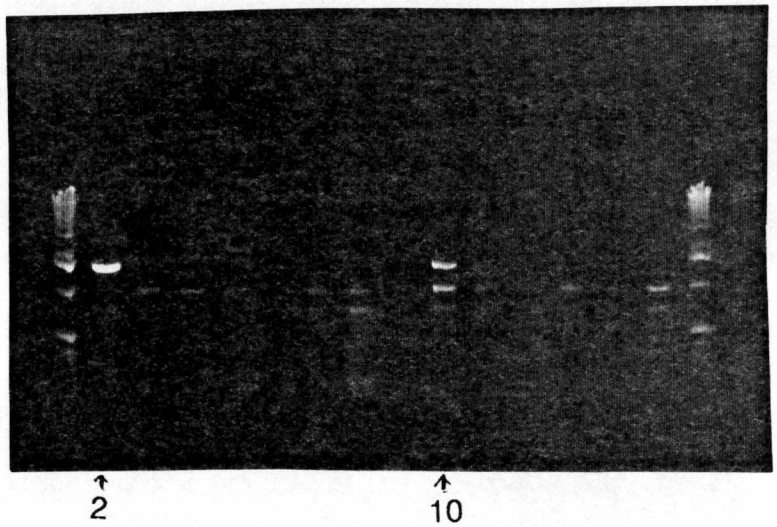


Figure 4.6 Electrophoresis of DNA generated by polymerase chain reactions of transformed pMAMneo-HE sense HRT-18 cells. Positive control reaction amplified supercoiled DNA of HE6 generating a 1.4 kb fragment (Lane 2). A PCR positive clone (Clone 8; Lane 10).

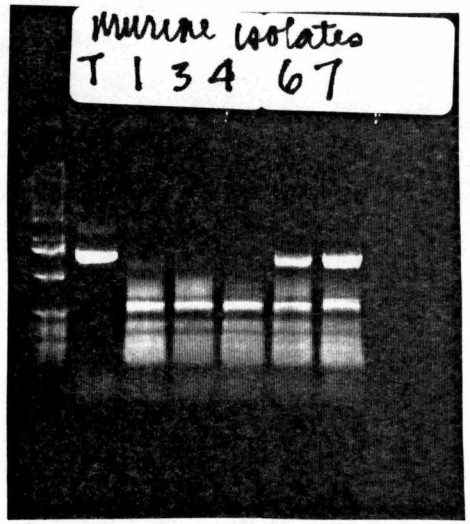


Figure 4.7 Ethidium bromide stained gel of DNA generated by polymerase chain reactions on transformed pMAMneo-HE sense L929 cells. Positive control reaction amplified DNA (Lane 2). Two PCR positive clones (Clone 6, 7; Lanes 6 and 7, respectively).

were stored as frozen cells. Clones 2, 6, 7, and 11 were used exclusively to evaluate expression of the HE gene in L929 cells, and it is the results of these analyses that are described below.

Southern Blot Analysis

Southern blots were performed with DNA generated from PCR reactions on cell lysates. The DNA was electrophoresed on a 1% agarose gel and stained with ethidium bromide to observe the amplified product (Figure 4.8A). After a record of the stained gel was obtained, the gel was fixed with alkali and neutralized as described for colony hybridization and the DNA was blotted onto a nylon membrane and probed with a $\gamma^{32}P$ end-labeled oligonucleotide specific for HE gene sequence. Southern blots were performed to serve two functions: to determine if the PCR generated fragment (1.4 kb) was HE gene specific and if the smaller molecular weight bands also generated by PCR of tranfected cell lysates also contained HE sequence. Positive control DNA used for each PCR reaction (ie., supercoiled plasmid HE6 DNA template and the described vector primers) served as positive controls for Southern blot analysis.

An HRT-18 clone (Clone 9) from the second transfection was determined to contain the HE gene by Southern Blot analysis. Clones 1, 2, 6, 7, and 11 from the third transfection of L929 cells were positive by Southern Blot analysis (Figure 4.8B). Probing the PCR generated fragments from transfected cell lysates, including the described lower molecular weight species, identified in a single band of approximately 1.4 kb. Therefore, the labeled HE specific probe did not hybridize to the smaller DNA fragments.

Hemadsorption

Transformed HRT-18 and L929 cells were induced 24 hours prior to evaluation by hemadsorption as described above. Following induction, it was

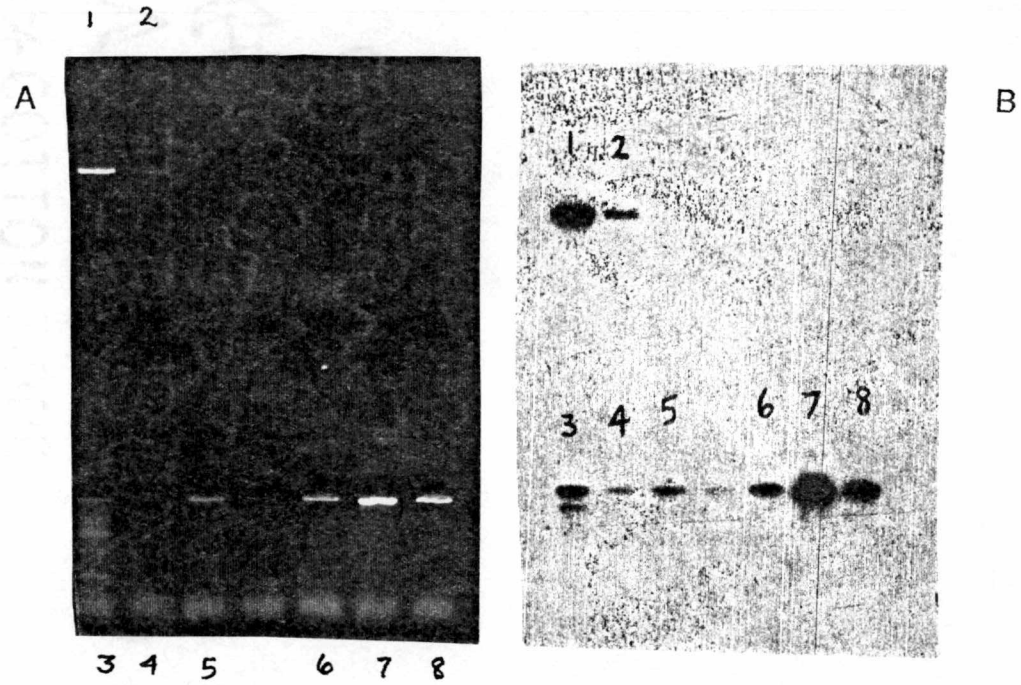


Figure 4.8 Ethdium bromide stained gel and Southern Blot of PCR products from transformed cells. (A) Agarose gel electrophoresis of PCR products from transformed cells. Positive control reactions (Lanes 1 and 8) and amplified HE gene products from HRT-18 cells (Lane 2) and L929 cells (Lane 3, 4, 5, 6, 7) are shown. (B) Southern blot analysis of PCR products blotted from the above gel (Figure 4.8A). Positive controls (Lanes 1 and 8) and positive clones (Lanes 2, 3, 4, 5, 6, 7) are shown.

observed that approximately 10% of the induced L929 cells contained spherical cytoplasmic vacuoles. Transformed HRT-18 cells occasionally contained similiar vacuoles, but never to the extent of the L929 cells (Figure 4.5B). Such vacuoles were rarely observed in the infected or uninfected control cells.

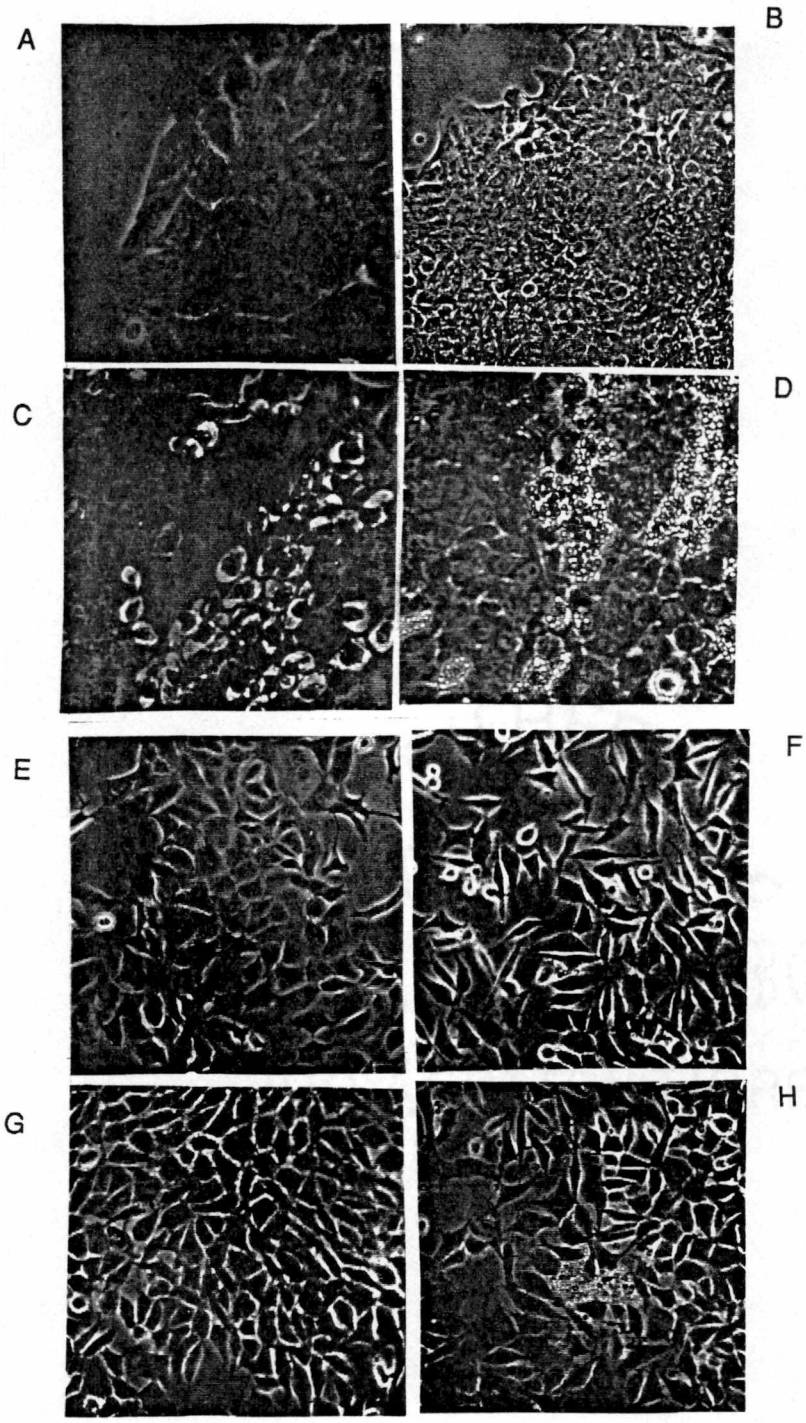
Murine erythrocytes were incubated with BCV-infected HRT-18 and L929 cells which served as positive controls for hemadsorption, and to uninfected HRT-18 and L929 cells, which served as negative controls. Both mammalian cell types showed round patches of adsorbed erythrocytes following incubation. Uninfected cells did not adsorb the murine red blood cells (Figure 4.9). Transformed HRT-18 and L929 cells containing the HE gene in both the sense and antisense orientations and pMAMneo vector failed to demonstrate hemadsorption (Figure 4.10).

Indirect Immunofluorescence

Since BCV-infected L929 cells were capable of hemadsorption albeit at a very low frequency, attempts were made to use them as positive controls for immunofluorescence. However, results using polyclonal Rabbit D antiserum on BCV-infected L929 cells were negative, probably because of the very small percentage of cells that became infected. Therefore, uninfected HRT-18 and L929 cells served as negative controls and BCV infected HRT-18 cells served as positive controls for all indirect immunofluorescence experiments.

Uninfected HRT-18 cells that were fixed for surface and internal fluorescence incubated with the anti-HE mAbs exhibited a slight background fluorescence that was limited to cellular junctions for surface fixation and within the cytoplasm of cells for internal fixation (Figure 4.11). Uninfected L929 cells exhibited a similiar pattern of fluorescence using the mAbs, but the intensity of background fluorescence was high enough to interfere with detection of low levels of expressed HE protein. Therefore, polyclonal antiserum was used for evaluating the L929 clones throughout. Polyclonal serum against the HE exhibited a similiar pattern of low

Figure 4.9 Hemadsorption results of bovine coronavirus infected and uninfected HRT-18 and L929 cells. (A) Uninfected HRT-18 cells, (B) Uninfected HRT-18 cells exhibiting nonhemadsorption, (C) BCV infected HRT-18 cells exhibiting cytopathic effect, (D) BCV infected HRT-18 cells exhibiting hemadsorption, (E) Uninfected L929 cells, (F) Uninfected L929 cells exhibiting nonhemadsorption, (G) BCV infected L929 cells, (H) BCV infected L929 cells exhibiting hemadsorption.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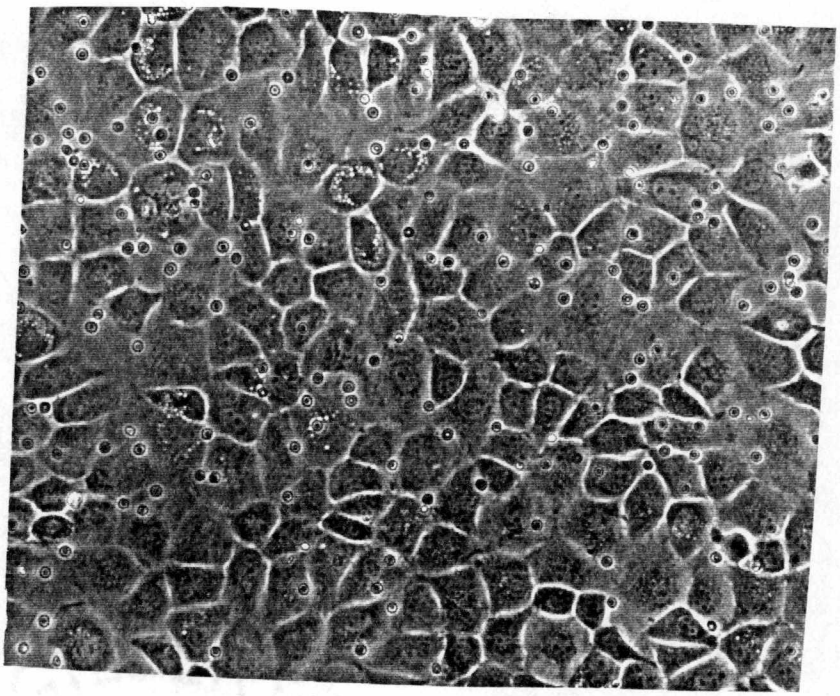

Figure 4.10 Nonhemadsorption of transformed L929 cells expressing the BCV hemagglutinin-esterase gene.

background fluorescence in uninfected HRT-18 (Figure 4.11) and L929 cells (not shown).

Transformed HRT-18 and L929 cells were induced as described 24 hours prior to fixation. Cells infected with BCV routinely demonstrated the cytopathic effects of infection (cytoplasmic vacuolization) at 24 hours post-infection.

Surface Expression of the Hemagglutinin-Esterase Protein

BCV infected HRT-18 cells fixed for surface fluorescence revealed strong surface expression of the HE protein (Figure 4.12; Panels B, D, F, G). Individual HRT-18 cells exhibited varying degrees of fluorescent intensity, but the general pattern was slightly granular, uniform in distribution and covered the entire cell surface. This pattern of fluorescence was seen in BCV infected HRT-18 cells using both polyclonal and monoclonal antibodies. All transformed HRT-18 and L929 cells were negative for surface expression of the HE protein regardless of the orientation of the HE gene and the antibody source (not shown).

Internal Expression of the Hemagglutinin-Esterase Protein

BCV infected HRT-18 cells fixed for internal fluorescence revealed strong cytoplasmic expression of the HE protein (Figure 4.12; Panels A, C, E, G). A reticulated pattern of fluorescence with increased perinuclear intensity was observed by both polyclonal and monoclonal antibodies.

HRT-18 and L929 cells transformed by the HE-sense construct revealed a low level fluorescence in the cytoplasm (Figure 4.13). The fluorescence pattern was also reticulated with the strongest fluorescence occurring around the periphery of the nucleus. Fluorescence appeared to be equally distributed among the majority of cells in a given field. The multinucleated and enlarged cells fluoresced with about the same intensity as single cells, however the pattern of fluorescence was

Figure 4.11 Surface and internal fluorescence of uninfected HRT-18 cells. (A) Internal fixation/Rabbit D, (B) Surface Fixation/Rabbit D, (C) Internal Fixation/mAb1, (D) Surface Fixation/mAb1, (E) Internal fixation/mAb2, (F) Surface fixation/mAb2, (G) Internal fixation/mAb3, (H) Surface fixation/mAb3.

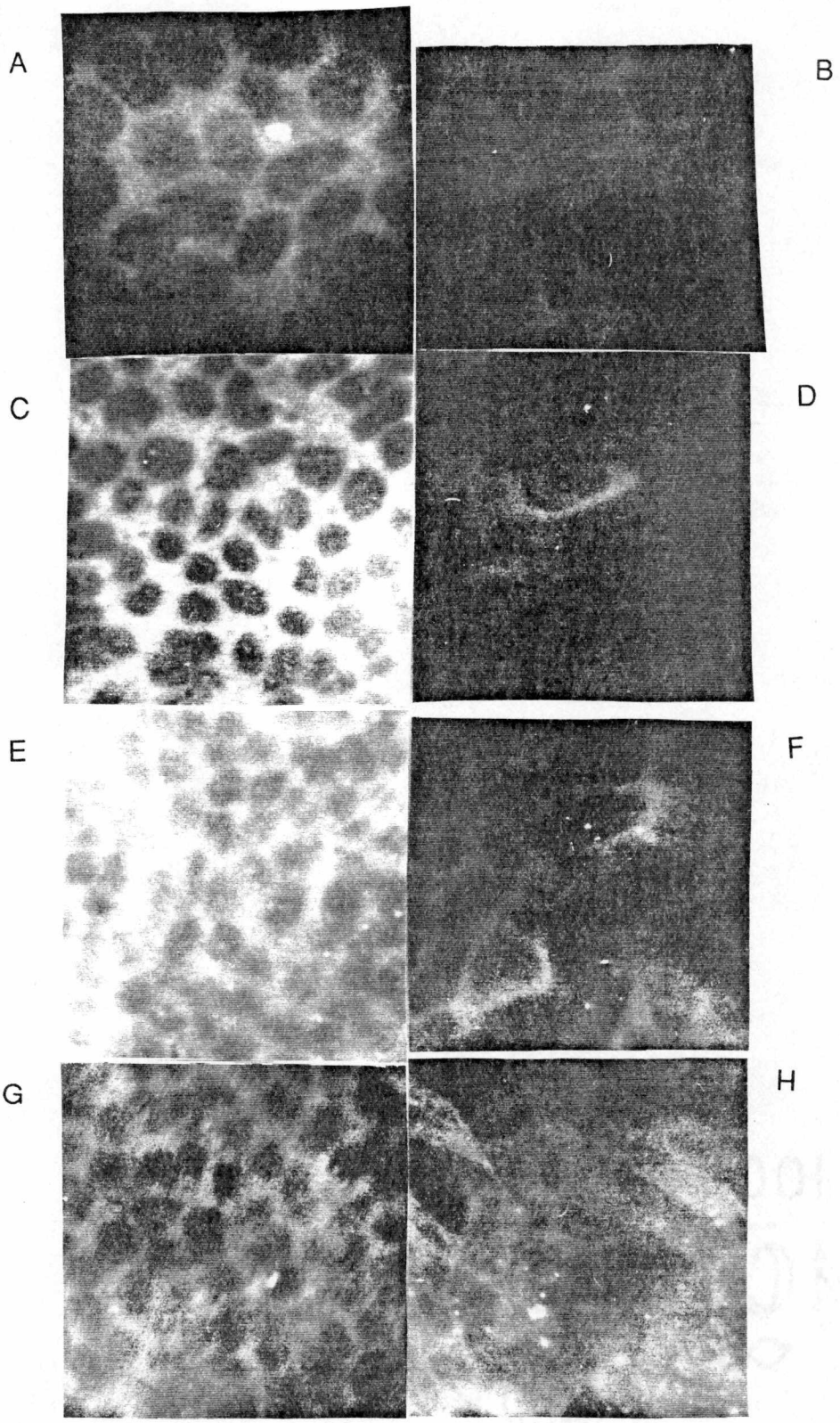
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Figure 4.12 Surface and internal fluorescence of BCV-infected HRT-18 cells. (A) Internal fixation/Rabbit D, (B) Surface Fixation/Rabbit D, (C) Internal Fixation/mAb1, (D) Surface Fixation/mAb1, (E) Internal fixation/mAb2, (F) Surface fixation/mAb2, (G) Internal fixation/mAb3, (H) Surface fixation/mAb3.

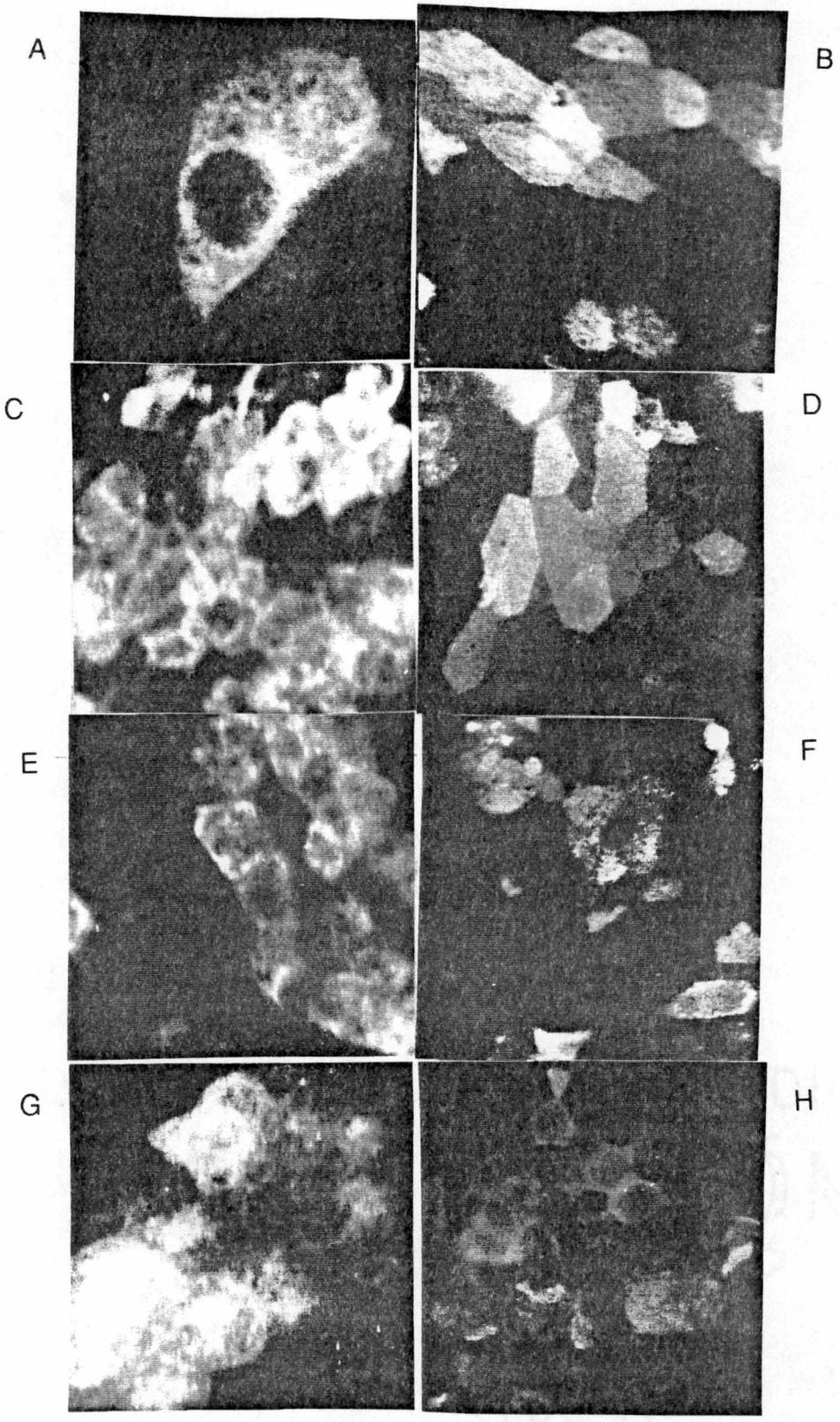
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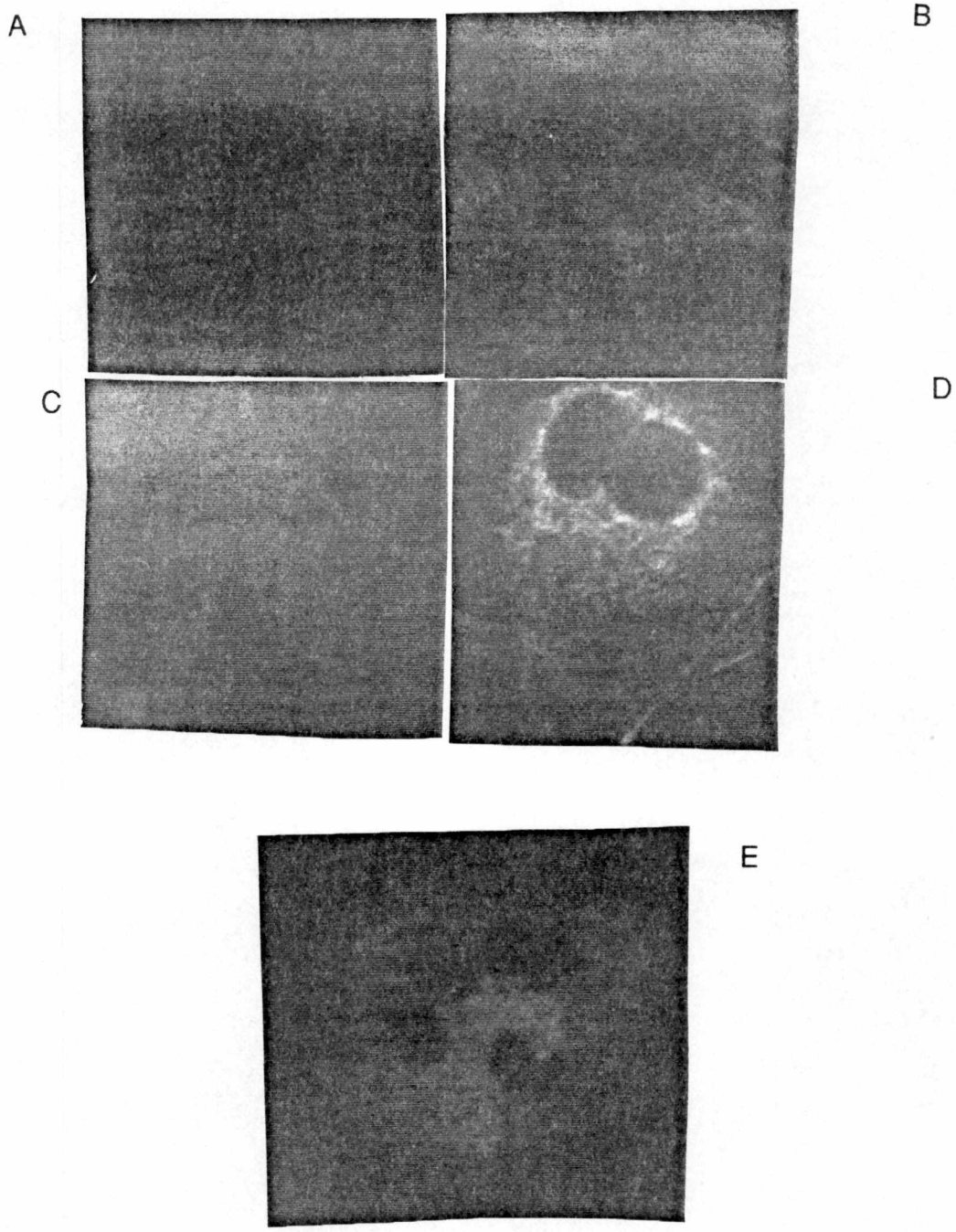
Figure 4.13 Internal fluorescence of transformed pMAMneo-HE sense L929 cells. (A) Untransformed L929 cells, (B) Clone 11, (C) Untransformed L929 cells, (D) Clone 2, (E) Multinucleated cell exhibiting granular pattern of internal fluorescence. Polyclonal Rabbit D serum was used in all panels.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more granular and more evenly distributed (Figure 4.13).

Transformed cells containing the antisense HE gene or the pMAMneo vector only had the same pattern of fluorescence as uninfected HRT-18 and L929 cells.

Immunoprecipitation

Immunoprecipitation was used to detect and characterize the HE protein expressed by the transformed HRT-18 and mouse L929 cells. Infected HRT-18 served as positive controls for the HE protein. The dimer form of the HE protein (140 kD) was routinely observed in ^{35}S labeled infected cell lysates precipitated with either the polyclonal or monoclonal antibodies. Adding β-mercaptoethanol to infected cell lysates prior to running the sample on a 8 or 10% polyacrylamide gel reduced the dimer HE protein to the monomeric form (65 kD; Figure 4.14). Since the HE protein is a glycosylated protein, the dimer and monomer forms precipitated as broad rather than sharp bands.

Control Experiments

The mAbs precipitated both the monomer and dimer of the HE protein from BCV infected HRT-18 cells (Figure 4.14: Control Gel A). Monoclonal Ab3 (Lanes 13 and 14) routinely precipitated less HE protein than did mAb1 and mAb2 (Lanes 9 and 10 ; Lanes 11 and 12, respectively) when the same amounts of labeled lysates were used. Interestingly, mAb1 also precipitated another protein from uninfected HRT-18 cells (Lanes 3 and 4). This cellular protein was estimated to be approximately 200 kD which reduced upon the addition of β-mercaptoethanol to a 90-95 kD species. Both the multimeric and monomeric forms of this cellular protein exhibited relatively sharp bands when compared to the precipitated HE protein.

To determine if mAb1 would precipitate a similiar cellular protein in L929 cells, lysates of ^{35}S labeled L929 cells tested. No such protein was observed (Figure 4.15: Control Gel B).

Figure 4.14 Control gel A for immunoprecipitates.

Lane 1, Rainbow Marker;
Lane 2, Ovalbumin;
Lane 3, Uninfected HRT-18/mAb1;
Lane 4, Uninfected HRT-18/mAb1+b-mercaptoethanol;
Lane 5, Uninfected HRT-18/mAb2;
Lane 6, Uninfected HRT-18/mAb2+b-mercaptoethanol;
Lane 7, Uninfected HRT-18/mAb3;
Lane 8, Uninfected HRT-18/mAb3+b-mercaptoethanol;

Lane 9, BCV Infected HRT-18/mAb1;
Lane 10, BCV Infected HRT-18/mAb1+b-mercaptoethanol;
Lane 11, BCV Infected HRT-18/mAb2;
Lane 12, BCV Infected HRT-18/mAb2+b-mercaptoethanol;
Lane 13 ,BCV Infected HRT-18/mAb3;
Lane 14, BCV Infected HRT-18/mAb3+b-mercaptoethanol.

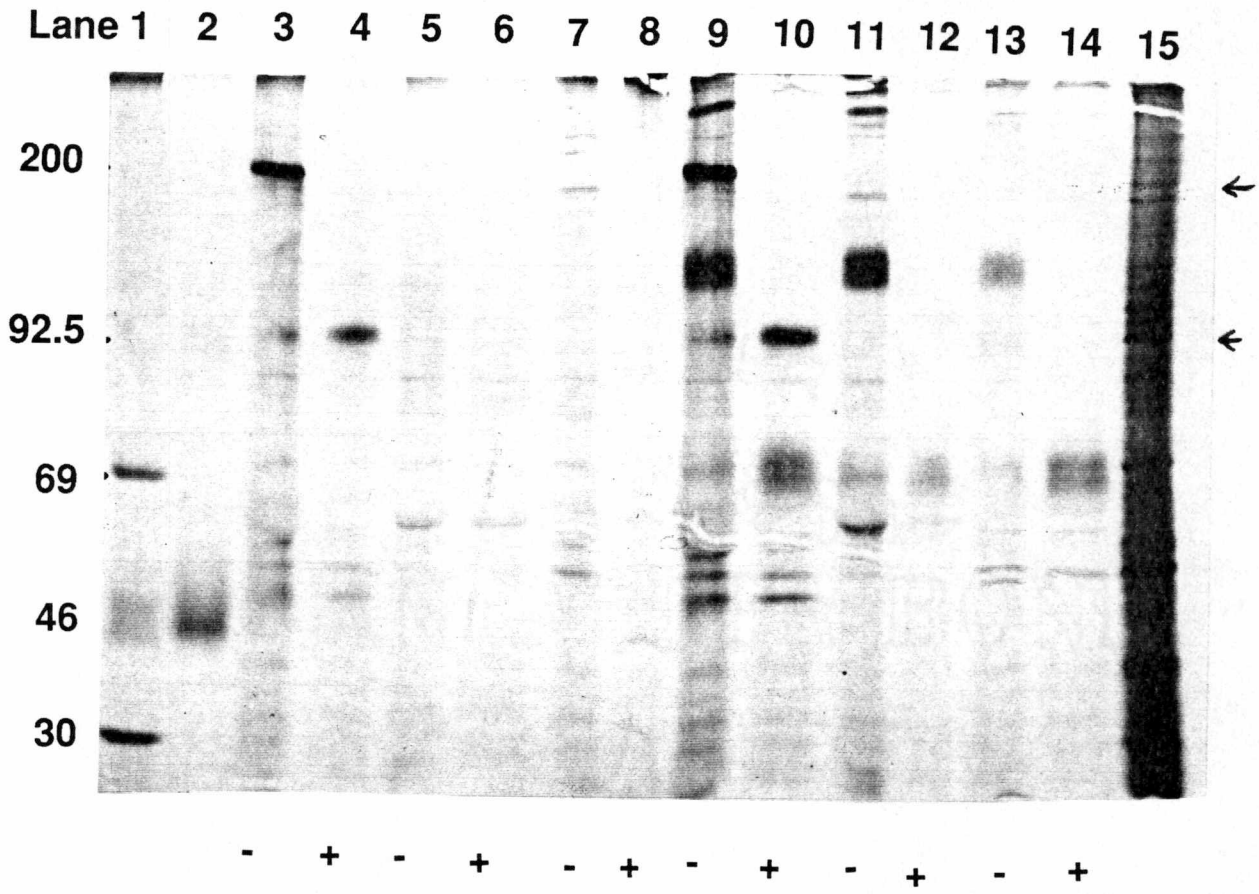
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
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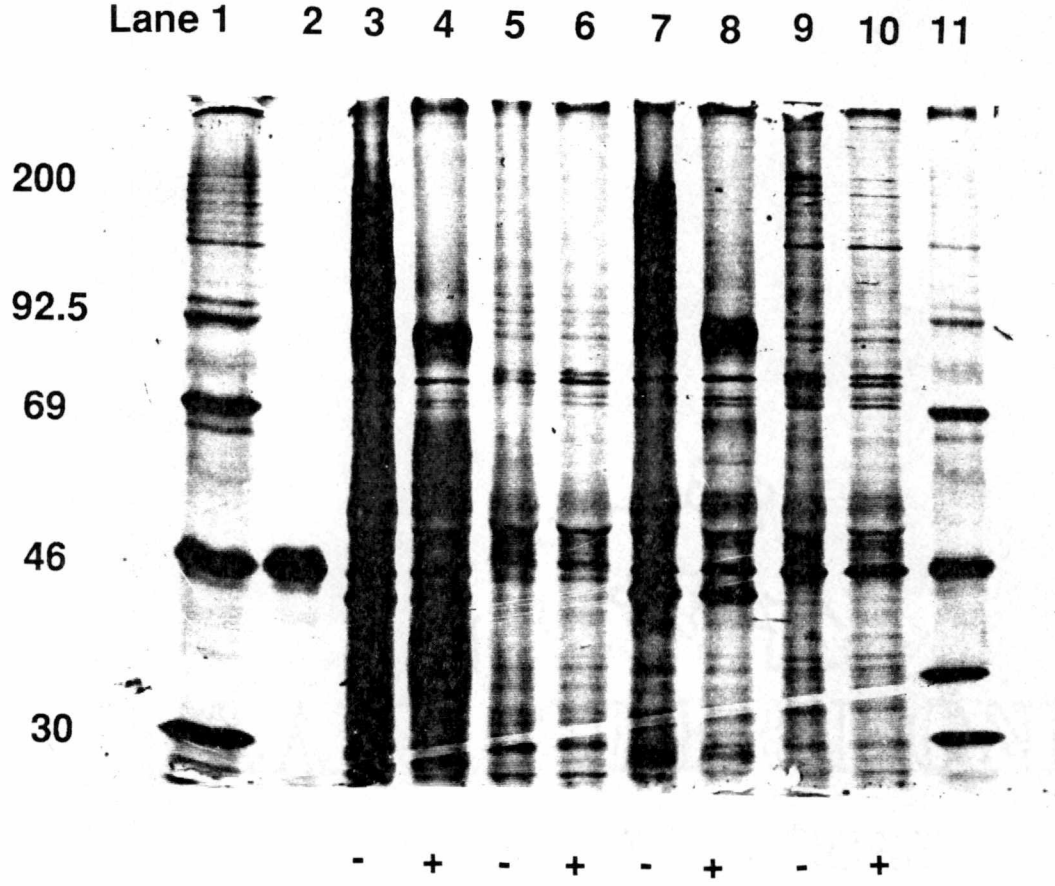
Figure 4. 15 Control gel B for immunoprecipitates.

Lane 1, Rainbow Marker;
Lane 2, Ovalbumin;
Lane 3, BCV Infected HRT-18/mAb1;
Lane 4, BCV Infected HRT-18/mAb1+b-mercaptoethanol;
Lane 5, BCV Infected L929/mAb1;
Lane 6, BCV Infected L929/mAb1+b-mercaptoethanol;
Lane 7, Uninfected HRT-18/mAb1;
Lane 8, Uninfected HRT-18/mAb1+b-mercaptoethanol;
Lane 9, Uninfected L929/mAb1;
Lane 10, Uninfected L929/mAb1+b-mercaptoethanol;
Lane 11, Rainbow Marker.


Lane 1
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Immunoprecipitation of Abberant Forms of the HE Protein

In general, maintaining equal numbers of cells between control samples and induced samples was difficult due to the slow growth rate of transformed cells. This problem was compensated for in subsequent experiments by either seeding larger Petri dishes with more transformed cells or growing the transformed cells to almost confluency prior to labeling (usually 5 to 7 days). This second method required that control dishes (uninfected HRT-18, infected HRT-18 and uninfected L929 cells) be seeded two days prior to labeling. Therefore, transformed cells were often older than cells used as infected controls.

At no time was a dimeric or monomeric protein of the proper size to be the glycosylated HE protein observed in any of the transformed cells. What appeared to be an abberant form of HE, however, was present in three separate clones of immunofluorescence-positive transformed L929 cells (Clones 2, 6, 11).

A sharp band of approximately 100 kD was precipitated from Clone 2 using mAb2 (Figure 4.16: Gel 1). The protein observed (lane 7) was not reduced by beta-mercaptoethanol (lane 8). Unfortunately, this gel did not contain an L929 control lane for comparison of cellular proteins precipitated by mAb2. However, polyclonal antiserum precipitated low levels of a protein of the same size in transfected L929 Clone 2 cells (Lanes 11 and 12), and failed to do so in untransformed cells (Lanes 5 and 6).

Cell lysates from L929 Clone 6 contained a similiar protein of 100 kD that precipitated with polyclonal Rabbit D antiserum that did not reduce in the presence of β-mercaptoethanol (Figure 4.17: Gel 2; Lanes 7 and 8; Lanes 9 and 10; and Lanes 11 and 12, respectively). Uninfected L929 cells did not show this protein (Lanes 13 and 14). Unifected and infected HRT-18 cells were used as negative and positive controls (Lanes 3 and 4 ; 5 and 6).

Immunoprecipitation of a later passage of L929 Clone 2 (Lanes 5 and 6) and Clone 11 (Lanes 11 and 12) revealed a nonreducible protein of approximately

60 kD (Figure 4.18: Gel 3). Clone 6 (Lanes 7 and 8) and Clone 7 (Lanes 9 and 10) show a faint precipitation product of approximately 100 kD that was not present in untransfected L929 cells (Lanes 13 and 14).

Figure 4.16 Experimental gel 1 for immunoprecipitates.

Lane 1, Rainbow Marker;
Lane 2, Ovalbumin;
Lane 3, BCV Infected HRT-18/mAb2;
Lane 4, BCV Infected HRT-18/mAb2+b-mercaptoethanol;
Lane 5, L929 Clone 2/mAb1;
Lane 6, L929 Clone 2/mAb1+b-mercaptoethanol;
Lane 7, L929 Clone 2/mAb2;
Lane 8, L929 Clone 2/mAb2+b-mercaptoethanol;
Lane 9, L929 Clone 2/mAb3;
Lane 10, L929 Clone 2/mAb3+b-mercaptoethanol;
Lane 11, L929 Clone 2/Rabbit D;
Lane 12, L929 Clone 2/Rabbit D+b-mercaptoethanol;
Lane 13, Untransformed L929/Rabbit D;
Lane 14, L929 Clone 2/mAb2 floating cells;
Lane 15, Rainbow Marker.

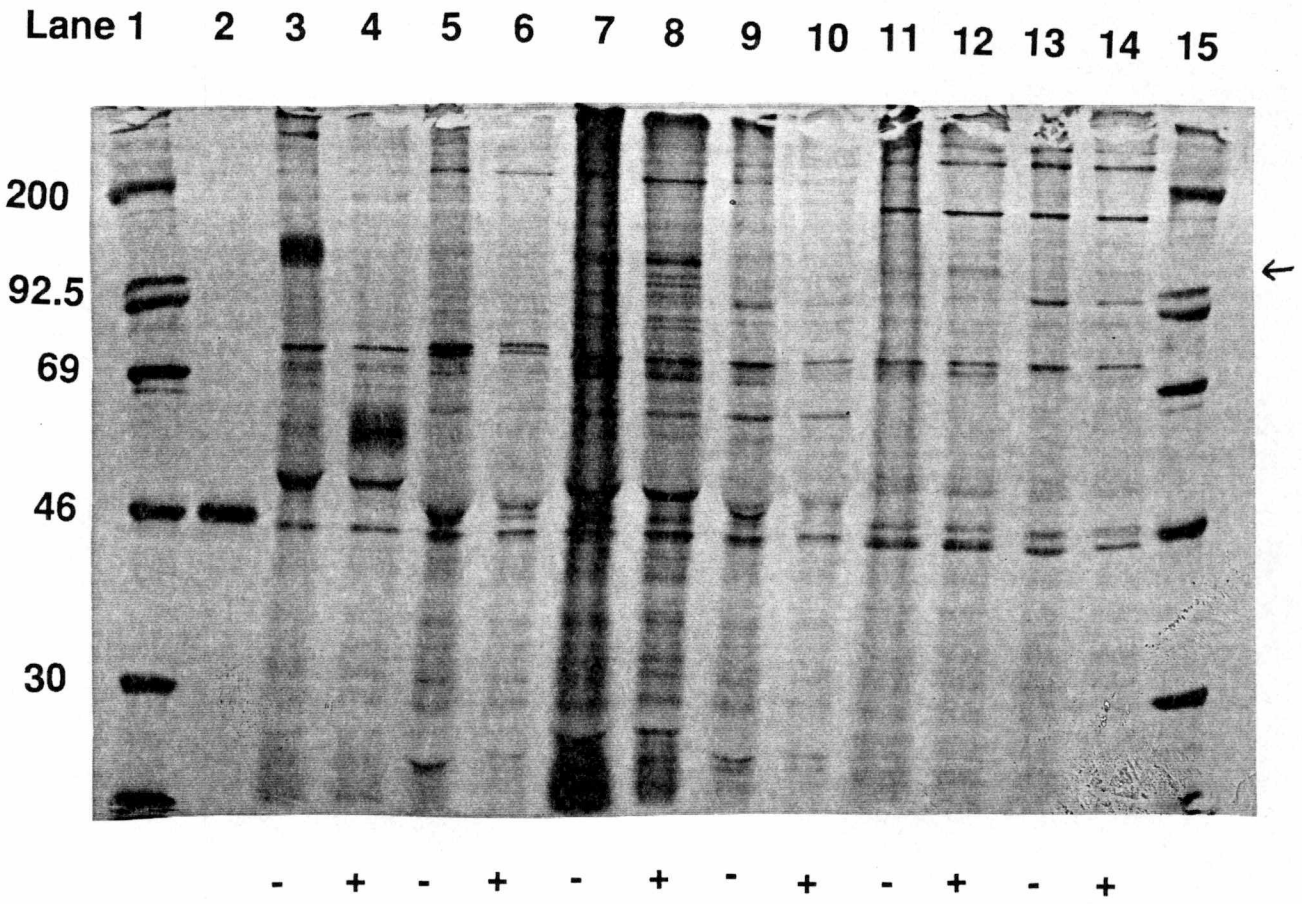
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Figure 4.17 Experimental gel 2 for immunoprecipitates.

Lane 1, Rainbow Marker;
Lane 2, Ovalbumin;
Lane 3, Uninfected HRT-18/Rabbit D;
Lane 4, Uninfected HRT-18/Rabbit D+b-mercaptoethanol;
Lane 5, BCV infected HRT-18/Rabbit D;
Lane 6, BCV infected HRT-18/Rabbit D+b-mercaptoethanol;
Lane 7, L929 Clone 6/Rabbit D;
Lane 8, L929 Clone 6/Rabbit D+b-mercaptoethanol;
Lane 9, L929 Flask B/Rabbit D;
Lane 10, L929 Flask B/Rabbit D+b-mercaptoethanol;
Lane 11, L929 Flask H/Rabbit D;
Lane 12, L929 Flask H/Rabbit D+b-mercaptoethanol;
Lane 13, Untransformed L929/Rabbit D;
Lane 14, Untransformed L929/Rabbit D+b-mercaptoethanol;
Lane 15, Rainbow Marker.

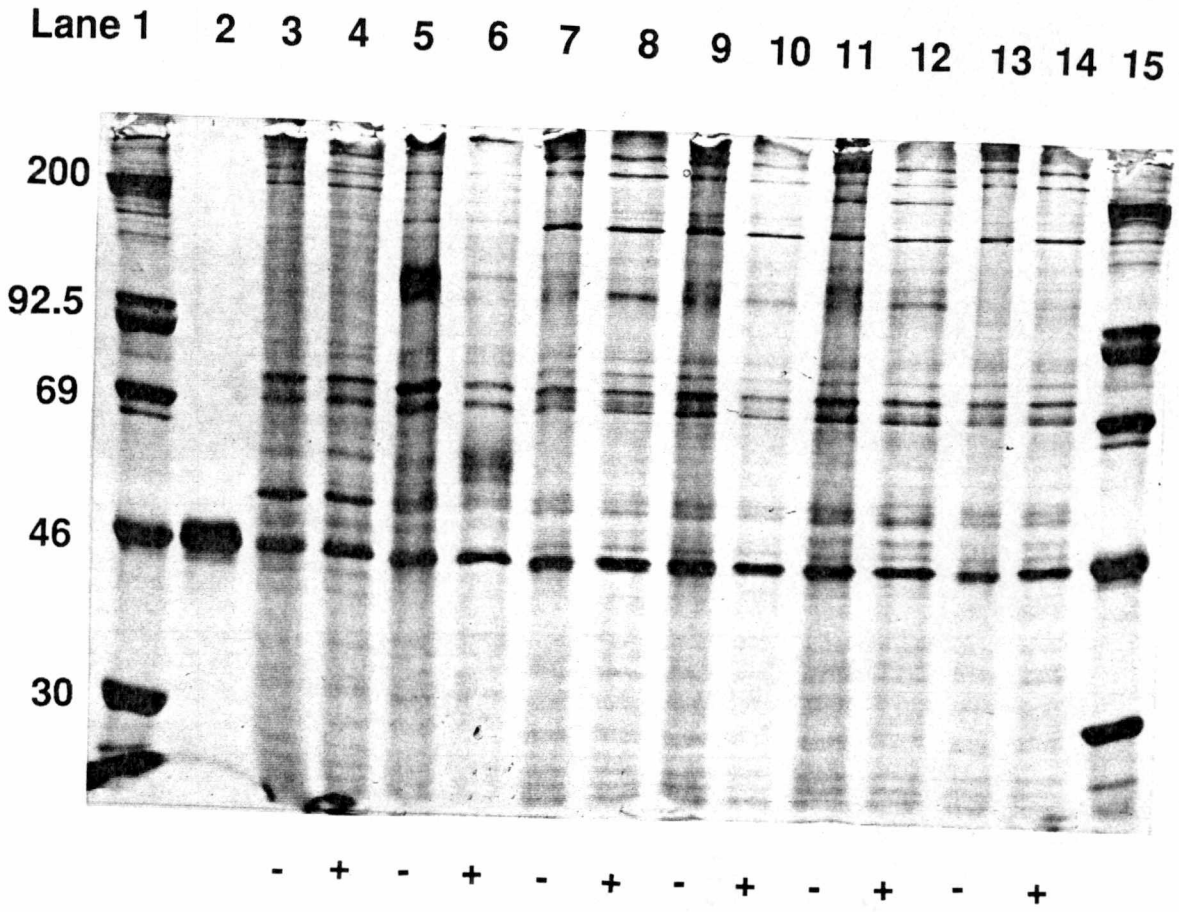
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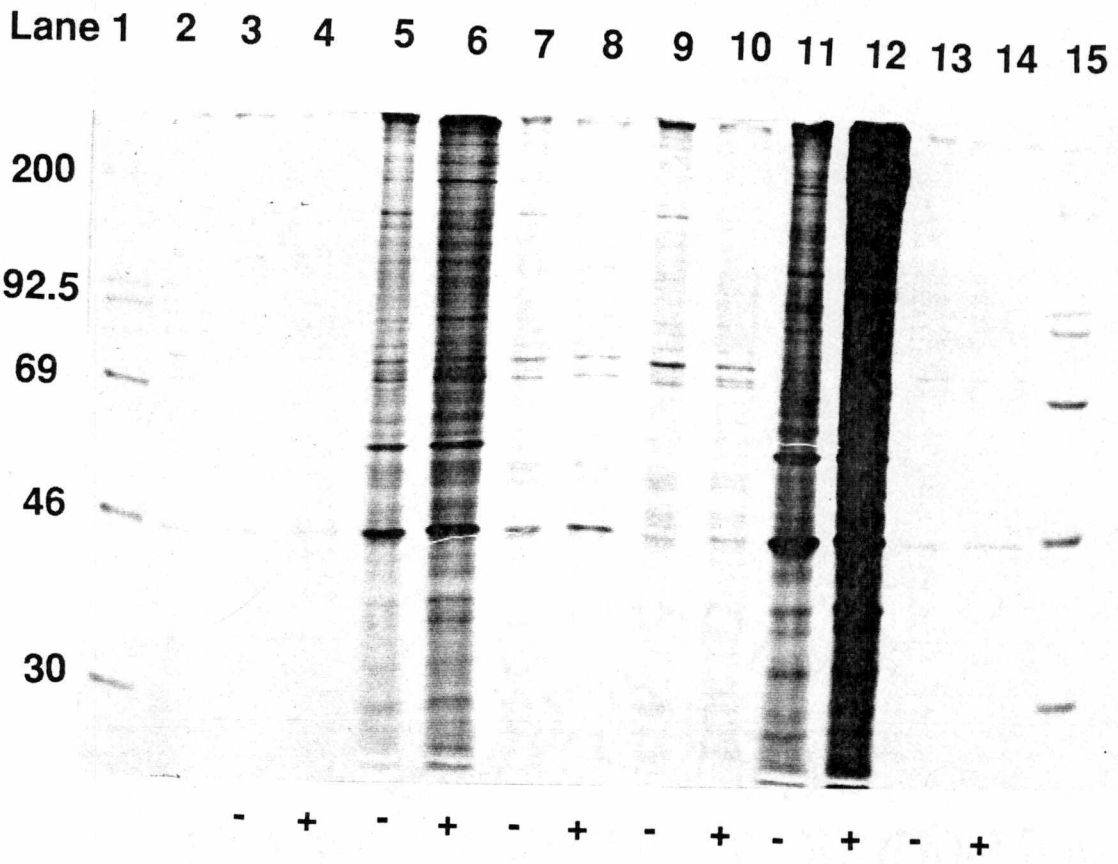
Figure 4.18 Experimental gel 3 for immunoprecipitates.

Lane 1, Rainbow Marker;
Lane 2, Uninfected HRT-18/Rabbit D;
Lane 3, BCV infected HRT-18/Rabbit D;
Lane 4, BCV infected HRT-18/Rabbit D+b-mercaptoethanol;
Lane 5, L929 Clone 2/Rabbit D;
Lane 6, L929 Clone 2/Rabbit D+b-mercaptoethanol;
Lane 7, L929 Clone 6/Rabbit D;
Lane 8, L929 Clone 6/Rabbit D+b-mercaptoethanol;
Lane 9, L929 Clone 7/Rabbit D;
Lane 10, L929 Clone 7/Rabbit D+b-mercaptoethanol;
Lane 11, L929 Clone 11/Rabbit D;
Lane 12, L929 Clone 11/Rabbit D+b-mercaptoethanol;
Lane 13, Untransformed L929/Rabbit D;
Lane 14, Untransformed L929/Rabbit D+b-mercaptoethanol;
Lane 15, Rainbow Marker.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Chapter 5
DISCUSSION

Possible Mechanisms As to the Origin of Expressed Mutated Forms of the Hemagglutinin-Esterase Protein

The hemagglutinin-esterase (HE) gene of the bovine coronavirus (BCV) was subcloned into a mammalian expression vector containing the inducible mouse mammary tumor virus promoter (MMTV). This recombinant DNA construct was transfected into two mammalian cell lines, human rectal tumor cells (HRT-18) and murine L929 cells, in order to create an inducible cell culture system in which the expression of the HE protein could be evaluated and possibly manipulated in future experiments.

Specific protein products of 100 kD and 60 kD were precipitated from cell lysates of transformed HRT-18 and L929 cells containing the HE sense orientation. These proteins were immunoprecipitated with a polyclonal antibody made against the viral gp 65 that routinely precipitated both the dimer (gp140) and monomer (gp65) forms of the viral HE protein in virus-infected cells. Electrophoresis of recombinant HE proteins revealed sharp bands that were not reducible by β-mercaptoethanol. A similiar product was not precipitated from transformed HRT-18 cells containing the antisense form of the HE gene nor from the expression vector without the HE insert. Therefore, it is hypothesized that these precipitated proteins are mutated forms of the HE protein.

Indirect immunofluorescence experiments, using the polyclonal that immunoprecipitated the putative mutated forms of the HE protein, detected a reticular pattern of fluorescence in L929 cells that had been transformed with the HE gene in the sense orientation. No surface fluorescence was observed. Positive internal fluorescence of the type observed suggests an internal accumulation of a protein product. This result supports the hypothesis of a

mutated form of the HE protein since the patterns of expression is very different from that in infected cells. If a signal sequence had been deleted from the HE gene, for example, a truncated protein may not have been properly processed and transported to the transfected cell surface.

Additionally, glycosylation or at least sugar processing may not have occurred since the bands seen by immunoprecipitation are sharp rather than diffuse. This observation suggest the presence of a single form of the mutated recombinant protein. Since the sugar moieties on the wild type HE protein are processed in the Golgi and hence become more heterogeneous in size (Hogue, 1989), a lack of glycosylation or sugar processing, or both, suggests the mutated protein may have become trapped in the endoplasmic reticulum of the cell.

No hemadsorption was ever observed on transformed HRT-18 or L929 cells containing the HE gene. This result is consistent with blockage of transport of a mutated HE protein to the cell surface.Transport to the cell surface may have failed because the primary or secondary structural requirements for a functional transport signal is missing. Perhaps multimerization (to a dimer or higher) is required for transport, but did not occur because of a missing primary or secondary structural feature. Conversely, the mutated protein may get to the surface but lack the proper primary or secondary structure necessary subsequent adsorption of murine erythrocytes.

Stable expression of the HE protein in this system required integration of the complete viral gene into the host cell genome, transcription of the gene, and translation of the message. The expressed protein must be stable and accumulate in sufficient quantities to be detected. These events can take place only if the recombinant gene and its product are compatible with the host cell.

Theoretically, subcloning the HE gene into a proven expression vector containing the MMTV promoter (Sardet, 1989) and transfecting the recombinant DNA into L929 cells should have resulted in a successful expressed protein. Instead, the expression of a viral gene was technically difficult and mutated forms

of the HE protein were expressed. These results cause one to speculate on the mechanisms behind expressing mutated forms of a viral glycoprotein.

The BCV HE gene has been expressed as a properly dimerized and surface expressed glycoprotein in cell culture using a transient expression assay. The HE was subcloned into a plasmid under the control of the T7 polymerase promoter and transfected into mammalian cells in which the T7 polymerase was expressed from an infectious vaccinia recombinant (Kienzle, 1990). Transient expression of the HE protein, in this case, did not require stable integration of the viral gene into the host cell genome. Therefore, stable expression of the BCV HE gene once incorporated into the nucleus may present a different scenario than transiently expressing this viral gene in mammalian cells.

The lack of a stably expressed a proper dimeric form of the HE in HRT-18 and L929 cells may be explained by two different mechanisms. (1) the HE gene may have been unstable in the chosen expression vector, and (2) the HE protein is cytotoxic when expressed in mammalian cells.

In considering the first hypothesis, the HE gene may have lost important DNA sequences during integration into the host cell genome or during replication. This hypothesis is supported by the observations made in which PCR positive clones became negative in subsequent PCR reactions. This phenomenon would require deletion of DNA sequences including at least one site of primer annealing or deletion of the entire HE gene including the vector's oligonucleotide annealing sites.

It is possible that intramolecular recombination may occur during replication thus deleting HE gene sequences (Wake, 1980; Roth, 1985). However, the exact mechanism of such a deletion in this system can only be speculated upon. However, computer analysis of messenger RNA secondary structure between the MMTV promoter and the 5' end of the HE gene is very high. These analyses are used for transcriptional analyses as cannot directly interpolated to evaluate DNA structure.

The second hypothesis which states that the HE protein is cytotoxic in a stable expression system is supported by two recent reports of viral proteins expressed in mammalian cells.

The hepatitis delta virus antigen (HDAg) gene in HeLa cells and HepG2, a hepatocyte derived cell line, is toxic to the cells (Macnaughton, 1990). The expression vector used in these experiments contained the Rous sarcoma virus promoter, hence the HDAg was being constituitively expressed. Several G418 resistant clones initially showed varying levels of HDAg expression by internal fluorescence and Western blots. However, a progressive loss of cells in cell culture was noted and those clones expressing high levels of HDAg could not be amplified. This phenonmenon was particularly noticable in HeLa cells (Macnaughton, 1990). Two clones expressing the HDAg were established in HepG2 cells. One clone showed expression of the HDAg in every cell, while the other clone never showed expression of HDAg, but remained G418 resistant. The expressed HDAg protein was determined to be a mutated form by the following analyses (1) the expressed HDAg was smaller in size that the viral HDAg as determined by cesium chloride gradient centrifugation, (2) the recombinant protein showed a nucleolar distribution by indirect immunofluorescence instead of the expected nucleoplasmic distribution, and the recombinant was not secreted from the host cell. Even though this study uses a constituitive promoter, these observations parallel the observations made while expressing the BCV HE gene in mammalian cells.

The MMTV promoter was used in an inducible expression system to express the Herpes simplex virus Type 1 glycoprotein gC1 (Friedman, 1989). Murine L cells (Ltk-) were tranfected with a recombinant containing the gC1 gene. A low level of gC1 mRNA was detected by immunofluorescence and Northern blot analysis in uninduced transfected cells. These data suggested that transfected cells were continuously expressing low levels of the recombinant gC1 protein. The authors of this study hypothesize that low levels of a cytotoxic

protein are enough to give a selective advantage to transfected cells not expressing the intact protein. Induction of the MMTV promoter by dexamethazone resulted in a 200 fold increase in gC1 specific mRNA as detected in transfected cells. Those cells expressing gC1 assumed "bizarre" syncytial shapes and death followed for most of the cells within 5 weeks. Additionally, clones expressing the gC1 gene under the control of a constituitive promoter (Moloney murine sarcoma virus) could not be established. The authors suggest that expression of Herpes simplex virus Type 1 gC1 is cytotoxic and requires an inducible expression system.

In our study, a low level of endogenous expression of the HE protein could theoretically occur due to the presence of steroids in growth medium supplemented with fetal calf serum. If the HE protein is toxic to the host cell, then low levels of HE protein may cause cell death. Surviving cells may have retained the G418 resistance gene with or without a truncated or abberant form of the HE gene.

Both of the above hypotheses can be tested by sequencing the DNA from clones expressing the truncated forms of the HE protein. Oligonucleotide primers are available in our lab that prime at different site along the HE gene (Figure 3.2). These primers could be used to sequence PCR amplified DNA to determine the exact deletions or other alterations within the HE gene construct. If deletions are similiar between different clones, then the deleted sequences may be the toxic portion of the protein.

Speculations on the Immunoprecipitated Protein Detected in Uninfected Human Rectal Tumor Cells

Immunoprecipitation of uninfected HRT-18 cell lysates by a monoclonal antibody (mAb1; also designated HC10-5; a gift from M. Parker) made against the hemagglutinin-esterase (HE) protein of BCV precipitated an unexpected multimeric protein. By comparison with marker proteins during electrophoresis, the multimeric form of the protein was approximately 200 kD which was reduced in the presence of β-mercaptoethanol to a species of approximate 90-95 kD. This size difference suggests that the multimer may be a dimer, but this remains to be confirmed. The intensity and sharpness of both the dimer and monomer bands suggest that this is an unglycosylated abundant protein in uninfected HRT-18 cells.

Two other HE specific monoclonal antibodies (mAb2, mAb3) and the polyclonal antibody used for immunoprecipitation did not precipitate this cellular protein from unifected HRT-18 cells. Thus, it can be concluded that mAb1 reacts with an antigenic epitope of an HRT-18 protein that is similiar to an antigenic epitope in BCV. This possibility has several interesting ramifications. It can be concluded that BCV shares an antigenic determinant with a component of a human cell line.

The cross-reactivity of a viral amino acid sequence with a host cell protein is termed "molecular mimicry" and this phenomenon has been previously described in other viral systems. The large T antigen of SV40, for example, has been shown to have common antigenic sites within host cells (Lane, 1980). The phospho-protein of measles (P3), the 140 kD protein of herpes simplex virus and the hemagglutinin of vaccinia all react with antibody epitopes that also react with subcellular intermediate filaments (Fujinami, 1983). A survey of more than 600 monoclonal antibodies to 11 different viruses found that 3% cross-reacted with

normal tissue (Srinivasappa et al., 1986). In most cases however, the actual sites of epitope similiarity between the virus and host cell is not known.

Does this molecular mimicry have any application to actual disease, or is it a molecular happenstance? As an example, myelin basic protein is a host protein that has been shown to share six amino acids with the hepatitis B virus polymerase (Fujinami, 1985). Rabbits vaccinated with a synthetic peptide composed of these similiar amino acids showed pathogenic lesions in their central nervous tissue.

This experimental allergic encephalomyelitis was theorized to be due to an immune response against a viral peptide that also induced a cross-reaction with a self-protein. Perhaps viral expression of a protein similar to a self-protein would increase tolerance of the host's immune system to a virus. This molecular mimicry would seem to contribute to establishing persistent viral infections.

A far reaching question may be asked concerning the precipitation of a human cell line protein by a viral envelope glycoprotein monoclonal antibody. What is the potential origin of the bovine hemagglutinin-esterase protein? Since coronaviruses bud through the intracellular membranes of the host during replication, could this virus have incorporated a host protein and incorporated an mRNA copy into its genome during its evolution?

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APPENDIX

APPENDIX

Preparation and Transformation of Competent E. coli Cells

Adapted from J.P. Weir's Lab (1989)

1. Inoculate 5 ml of YT medium (without ampicillin) with a single colony of E. coli (HB101's) taken from a freshly streaked plate incubated overnight.

2. Incubate medium at 37°C until it is turbid.

3. Inoculate 100 ml of prewarmed YT (37°C) with the 5 ml culture.

4. Incubate medium at 37°C until the OD=0.5. This step is critical and length of incubation can vary dramatically.

5. Collect medium into 50 ml Falcon tubes and spin cells at 3000 rpm/15 min/4°C.

6. Resuspend cells in 33 ml of RF1 Solution* and mix well. Incubate cells on ice for 20 min.

7. Resuspend cells in 5 ml of RF2 Solution** and incubate them on ice for 15 min.

8. Mix cells gently and distribute them into sterile chilled 1.5 ml microfuge tubes (Eppendorfs) in 200 µl aliquots.

9. Flash freeze cells on dry ice with caps open. When cells are frozen, close caps and store at -80°C.

*RF1 (Make up in 100 ml amounts and keep at 4°C).

RbCl	1.2 grams
MnCl-4H2O	.99 grams
K-Acetate	3.0 ml of a 1 M stock (pH=7.5)
CaCl2-2H2O	.15 grams
Glycerol	15.0 grams

Add glycerol last by placing bottle on the balance and adding it directly to the solution. Adjust the pH to 5.8 with glacial acetic acid. Bring to 100 ml with sterile H_2O. Sterilize solution by filtration.

**RF2 (Make up in 100 ml amounts and keep at 4°C).

MOPS	2.0 ml of a 0.5 M stock (pH=6.8)
RbCl	.12 grams
$CaCl_2$-$2H_2O$	1.1 grams
Glycerol	15.0 grams

Adjust pH to 6.8 with NaOH. Sterilize by filtration.

For good competent cells keep cells cold at all times including centrifugation.
Be gentle during resuspension and mixing.

3.2 Transfection Procedure

(Adapted from Lipofectin^R Reagent Insert, Product Bulletin #18292B, BRL, Inc.)

1. Seed cells in 25 cm2 tissue culture flasks in 10% FCS/DMEM as described elsewhere. Incubate at 37°C until cells are 80-85% confluent.
2. Dilute 20 µg of clean DNA (range 1-20 µg) to a volume of 50 µl with sterile H_2O.
3. Dilute 35 µg of Lipofectin^R (range 20-40 µg) to a volume of 50 µl with sterile H2O in a polystyrene tube. Mix well.
4. Combine the DNA:H_2O and Lipofectin^R:H_2O. Mix gently and do not vortex.
5. Let stand at room temperature for 15 min. Solution should be cloudy but without a visible precipitate.
6. Pour off 10% FCS/DMEM from cells. Wash cell in flask with 3.0 ml of OptiMem^R Reduced Serum Medium (Gibco).
7. Pour off OptiMemR and replace with 3.0 ml fresh OptiMemR.
8. Add DNA:LipofectinR dropwise with a sterile pipette.
9. Rock flask gently and maintain it in a horizontal position.
10. Incubate cells for 5 to 24 hours at 37oC.
11. Add 3.0 ml 10% FCS/DMEM to cells (do not pour off OptiMemR). Reincubate cells for another 24 to 72 hours. Check cells for viability to determine reincubation time.
12. Split cells as described elsewhere and reseed for selection of transformants.

3.6 POLYMERASE CHAIN REACTION USING CELL LYSATES

(Adapted from Smithies, PNAS. 1988)
As communicated by Bill Lapps

1. Isolate 1-10,000 cells. (Use one drop of cells split 1:5 from a 25 cm^2 flask)
2. Add 5 µl phosphate buffered saline.
3. Add 20 µl H_2O.
4. Incubate 90-95°C for 10 min.
5. Centrifuge briefly in microfuge to spin down condensate.
6. Add 10 µg Proteinase K from frozen stock solution (1 µl of a 10 mg/ml stock)
7. Incubate 55°C for 30 min to 1 hour.
8. Centrifuge briefly.
9. Boil 90-95°C for 10 min to inactivate Proteinase K. Centrifuge briefly.
10. Aliquot out 25 µl of PCR mix* into small PCR tubes (Robbins Scientific).
11. Add 25 µl of cell supernatant to PCR mix. Avoid pellet at bottom of tube.
12. Add 0.2 µl of Taq I enzyme (Promega).
13. Amplify 30 cycles under appropriate conditions**.
14. Remove 10 µl from tube and run on a 1% agarose gel stained with EtBr and view under UV illumination.

PCR MIX* (recipe is for 20 samples)

dNTPS (125 mM each)	160 µl
Promega 10X Buffer	100 µl
dH_2O	220 µl
Primer A	10 µl
Primer B	10 µl

May be frozen as a stock solution without Taq I.

**For a 1.6 kb fragment amplification parameters:

Denaturation (95°C):	30 seconds
Annealing (55°C):	30 seconds
Extension (72°C):	1 min 30 seconds

VITA

Joanne L. Maki was born in Philadelphia, Pennsylvania on September 7, 1957. She received a Bachelor of Science degree cum laude from West Virginia University in Morgantown, West Virginia in 1979. She received a Doctorate of Veterinary Medicine from Louisiana State University School of Veterinary Medicine in Baton Rouge, Louisiana in 1983. She entered the graduate program at the University of Tennessee School of Veterinary Medicine, Knoxville, Tennessee in the spring of 1987 to complete a Master's Degree in Comparative and Experimental Medicine in May 1991. Joanne has accepted a position with the veterinary pharmaceutical company, Rhone-Merieux, Athens, Georgia.