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Heterogeneity of respiratory syncytial virus G glycoproteins : antigenic and molecular analyses

Robert Bruce Duncan

To the Graduate Council:

I am submitting herewith a dissertation written by Robert Bruce Duncan entitled "Heterogeneity of respiratory syncytial virus G glycoproteins : antigenic and molecular analyses." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Michael A. Breider, Major Professor

We have read this dissertation and recommend its acceptance:

Leon N. D. Potgieter, J. Erby Wilkinson, Barry T. Rouse

Accepted for the Council:

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Vice Provost and Dean of the Graduate School

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Associate Vice Chancellor
and Dean of The Graduate School

HETEROGENEITY OF RESPIRATORY SYNCYTIAL VIRUS G GLYCOPROTEINS: ANTIGENIC AND MOLECULAR ANALYSES

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Robert B. Duncan Jr.

December 1991

DEDICATION

This dissertation is dedicated to my parents

Mr. Robert B. Duncan

and

Mrs. Georgeanne S. Duncan

who have freely given their love, kindness, and understanding and provided me guidance, resources, and invaluable educational opportunities,

and to my wife

Susan E. Duncan

who is my loving companion and partner in the future.

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ABSTRACT

Classification of respiratory syncytial viruses into subgroups is based primarily on genetic and antigenic divergence of an important immunogenic envelope glycoprotein that may be important in disease immunopathogenesis and immunoprophylaxis. Currently, there are two human respiratory syncytial virus (HRSV) subgroups, but relatively little is known about bovine, caprine, and ovine respiratory syncytial virus isolates. Laboratory work was conducted in 1989-1991 to gather data on subgroup divergence and G glycoprotein heterogeneity among respiratory syncytial viruses. Seven bovine (BRSV 375, 391-2, FS1, NMK7, 1143, 1144, and 16186), two human (HRSV A2 and B8/60), one caprine (CRSV), and one ovine (ORSV) respiratory syncytial virus strains from various geographic sources were analyzed using current biomolecular techniques including Western blot analysis, polymerase chain reaction, molecular cloning, dideoxy nucleotide DNA sequencing, and RNase mismatch cleavage. Western blot analysis was performed using bovine anti-BRSV strain 375 and human anti-HRSV sera. The bovine antiserum cross-reacted with the G glycoproteins from all BRSV strains tested, whereas the human antiserum reacted with the G glycoprotein from HRSV A2. Neither antiserum cross-reacted with the G glycoproteins from HRSV strain B8/60, CRSV, or ORSV. Oligonucleotide primers based on the nucleotide sequence of the BRSV strain 391-2 G glycoprotein gene were used in the polymerase chain reaction to amplify virus-specific G glycoprotein gene cDNAs from genomic RNA of CRSV and BRSV strains 391-2 and 375. Virus-specific G glycoprotein gene cDNA could not be amplified from ORSV genomic RNA. Nucleotide sequence of glycoprotein G gene cDNA from BRSV strain 375 was compared to a similar sequence from

BRSV strain 391-2 indicating 95.0% and 89.9% nucleotide and amino acid identities, respectively. A radiolabelled riboprobe was made from a cDNA copy of the BRSV strain 375 G glycoprotein gene and all virus strains were compared by RNase mismatch cleavage analysis. The data indicated a close genetic relationship among CRSV and all BRSV strains suggesting that these strains be included in the same subgroup. In agreement with previously published data, the HRSV strains tested belong to additional RSV subgroups. Additionally, ORSV likely should be classified in a separate RSV subgroup.

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PART 1

GENERAL INTRODUCTION AND OVERVIEW

CHAPTER 1

LITERATURE REVIEW

Historical Perspectives

Human respiratory syncytial virus (HRSV) was first isolated in 1956 from a laboratory chimpanzee during an outbreak of illness that resembled a common cold (75). Hence, it was first named chimpanzee coryza agent (75). Soon after the isolation of the chimpanzee virus, an identical virus was isolated from a child with pneumonia and again from a child with croup in Baltimore, Maryland (75). Serologic studies revealed that most children in the Baltimore area became infected with the agent before four years of age (75). Similar investigations indicated that HRSV was associated with lower respiratory tract disease in humans and that it occurred worldwide (75). This agent is now recognized as the most important viral cause of lower respiratory tract disease in infants and children; its name was subsequently changed from chimpanzee coryza agent to human respiratory syncytial virus (75). The new name reflected its human origin and its characteristic cytopathic effect on cells in cell culture; and formation of large multinucleated cells (syncytia) as the result of cell to cell fusion (75).

In 1968, discovery of a specific neutralizing antibody in bovine serum against HRSV led to speculation that cattle harbored a virus antigenically related to HRSV (28). The first reported isolation of bovine respiratory syncytial virus (BRSV) was in 1970 (92) from a respiratory disease outbreak in cattle in Switzerland, and subsequently, BRSV was isolated from cattle in Japan (48). The first isolation of BRSV in the United States was in Iowa and Missouri in 1974 (100,106). Global distribution of BRSV is supported by its isolation and corresponding serologic

evidence from cattle during acute respiratory disease outbreaks worldwide (30,50,60,61,124). Caprine respiratory syncytial virus (CRSV) was isolated from a herd of pygmy goats with acute respiratory disease in 1979 (107). In the mid 1970's, serologic studies in sheep revealed BRSV cross-reactive antibodies (13,106) lending to speculation that sheep might have an antigenically related, but distinct respiratory syncytial virus (RSV) (106). Isolation of a respiratory syncytial virus from an adult sheep with rhinitis was reported in 1983 (64). In addition to humans and cattle, serum antibodies to respiratory syncytial virus have been detected in cats, dogs, pigs, sheep, and goats (8).

Clinical and Epidemiological Perspectives

Human respiratory syncytial virus is a major cause of serious respiratory infection in infants and children (43). It is the main cause of bronchiolitis and pneumonia in children less than six months of age (80). Approximately 40-50% of children hospitalized with bronchiolitis and 25% of children hospitalized with pneumonia are the direct result of HRSV infections (43). Yearly hospitalization costs attributed to this disease are approximately $300,000,000 and the average annual number of hospitalized cases is approximately 91,000 (43).

HRSV infection typically occurs as annual outbreaks in the winter and early spring in temperate climates and is highly contagious among infants and children (81). Annual epidemics are signalled by an increase in the number of children admitted to hospitals for bronchiolitis and pneumonia (81). HRSV infection in infants between the ages of six weeks and six months typically causes upper respiratory symptoms, however, in 25-40% of such infections, the lower respiratory tract is also involved. In these infants, mild disease is characterized by rhinorrhea,

cough, and fever. Anorexia and sneezing may also be seen. In more severe cases, coughing and wheezing progress to dyspnea and severe tachypnea; and in advanced disease, hypoxia becomes more extreme and listlessness and apnea occur (75). Recurrent infections are common although they tend to progressively diminish in severity (55). HRSV also can cause respiratory disease in young adults (34), adults (119), and the elderly (37,55) and can be severe in the latter (55). In older children and adults, HRSV infections usually cause upper respiratory symptoms that can be moderately severe (34), although, illness as a result of reinfection in adults seems to be less severe (119).

Recent estimates of economic losses due to respiratory disease in the United States cattle industry range between 250 and 750 million dollars annually (4,72). BRSV is a major emerging pathogen of cattle and is recognized as an important cause of respiratory tract disease in nursing beef calves, feedlot calves, and dairy calves (5-8,14,20,35,38,42,73,111). Outbreaks tend to be seasonal, occurring in the fall and winter, yet outbreaks during other parts of the year may occur (8). BRSV affects all age groups of cattle including calves (32,67,95,100), weaned calves (35,38,106) and adults (48,69,92). Respiratory tract disease associated with BRSV in weaned calves typically is associated with acute onset of fever followed by increased respiratory rate, serous ocular and nasal discharge, salivation, anorexia, and depression (5,8). The disease progresses to include severe dyspnea and occasional development of submandibular and cervical edema and subcutaneous emphysema. Varying degrees of subpleural and interlobular pulmonary edema and emphysema may develop. Pleuropneumonia, when present, often is the result of secondary bacterial infection. Histopathologic features of the disease include syncytial cell formation in the bronchiolar and alveolar epithelium, intracytoplasmic

inclusion bodies, proliferation and/or degeneration of the bronchiolar epithelium, alveolar type II pneumocyte proliferation, alveolar edema and hyaline membrane formation.

Respiratory tract disease associated with HRSV and BRSV resemble each other closely, particularly in infants and children where annual winter outbreaks are similar (55). Most severe disease is seen in children and calves between one and three months old when RSV-specific maternal antibodies are usually present (55). Reinfections are common and are accompanied by progressively milder symptoms in children and are symptomless in calves (55).

Respiratory syncytial virus in other ruminants produces similar clinical disease as BRSV or HRSV. Caprine respiratory syncytial virus (CRSV) was isolated from a herd of pygmy goats that had fever, anorexia, malaise, cough, mucopurulent nasal discharge, purulent lacrimal discharge, and photophobia (107). Ovine respiratory syncytial virus (ORSV) was isolated from a yearling ewe with rhinitis (64). Experimental inoculation of lambs with ORSV caused mild conjunctivitis and slight accumulation of alveolar macrophages and neutrophils in alveoli and bronchioles (33).

Relatively little is known about interspecies transmission of RSV strains. Experimentally, HRSV produces mild clinical disease in calves (51,114), but BRSV is not known to produce disease in humans (55). Sheep can be experimentally infected with BRSV (1,2,26,65,116,117) and have served as a model for BRSV infection. Both calves and deer were infected experimentally with ORSV (17).

Respiratory Syncytial Virus Characteristics

Virus Morphology

HRSV is pleomorphic and ranges from 150-300 nm in diameter (75). Individual virions are membrane-bound particles with short closely spaced projections (75). The lipid bilayer membrane encloses a RNA nucleocapsid that contains a non-segmented, negative-sense, single strand of RNA (75). The ribonucleoprotein of the nucleocapsid has a herring-bone appearance when viewed by negative-stain electron microscopy (75). Progeny virions mature by budding from the plasma membrane of the cell (75).

BRSV also has been characterized ultrastructurally (49). In ultrathin sections, mature virus particles, measuring 80-130 nm in diameter, were seen budding from the plasma membrane (49), while in negative stain preparations, virus particles were spherical and 80-450 nm in diameter. Ultrastructural similarities between BRSV, HRSV, and pneumonia virus of mice (PVM) support a similar classification for these three viruses (49).

CRSV ultrastructural morphology is similar to HRSV and is characterized by spherical particles of 90-160 nm and variably long filamentous forms 100-160 nm in diameter (66,107). Trudel, et al examined thin sections and negatively stained preparations of HRSV, BRSV, and CRSV that had been propagated on an ovine kidney cell line (118). Morphologic features of the three virus strains were similar. Negatively-stained preparations revealed that virions of the three strains were usually pleomorphic, spherical, and typically measured 100-450 nm in diameter. If viral particles were fixed in glutaraldehyde before negative staining, rod-shaped particles and terminal blebs were seen. Ultrathin sections of HRSV, BRSV, and

CRSV were similar. Variably long filamentous particles had constant diameters of 95±5 nm, but up to 80% of the CRSV and BRSV particles were linked in uniquely distinct networks by well defined bridges 12±3 nm in length and 17±1 nm in diameter, whereas HRSV had no bridges. The authors suggested that this morphologic difference might distinguish HRSV from BRSV and CRSV. In contrast to HRSV, BRSV and CRSV, the ultrastructure morphology of ORSV has not been characterized.

Properties of the Virus

Most of the molecular characterization of the pneumovirus genus has been done with HRSV. Detailed molecular analyses of cDNA clones from HRSV mRNAs have led to identification of ten virus-specific mRNAs that code for ten unique polypeptides (23). Nucleotide sequences for nine of the ten genes are known (69). In contrast, the molecular characterization of BRSV, CRSV, and ORSV is much less complete than that of HRSV. Few studies have compared the structural proteins of BRSV, CRSV, and pneumonia virus of mice (PVM) to HRSV (3,19,81,91,118). The structural proteins of ORSV have not been characterized. Recently, detailed antigenic and genetic analyses of BRSV have been compared and contrasted to HRSV (68,69). These data indicate that BRSV should be classified in a subgroup separate from HRSV subgroups A and B (68,69). The precise relationship between CRSV or ORSV and BRSV or HRSV is less clear, because few comparative studies have been done.

Genomic Organization and Characterization

The genome of HRSV is a single negative-sense strand of RNA composed of approximately 15,000 nucleotides (24). It contains ten genes that are transcribed as a single unit from a promoter at the 3' end of the genome (27). Ten unique mRNAs are transcribed and each encodes a unique protein (47). The order of gene transcription from the 3'terminus is 1C, 1B, N, P, M, 1A, G, F, 22k, and L (24) (see Figure 1).

Viral Protein Characteristics

Table 1 outlines the RSV genes and their products and functions. Figure 1 schematically depicts the virion structure and the organization of the viral genome. Of the ten viral proteins; only 1B and 1C are nonstructural (75). The nucleocapsid is composed of the viral genomic RNA, nucleocapsid (N) protein, phosphoprotein (P), and the large protein (L) (75). N is an abundant protein and serves as the main structural protein of the nucleocapsid (75). The function of the P and L proteins is unknown, however, they probably are components of the polymerase complex and function in transcription and replication of viral RNA (75).

The major glycoprotein (G), fusion protein (F), 1A protein, and 22k protein are associated with the cellular membrane and the virus envelope (75). The G, F, and 1A proteins are glycosylated (55), whereas 22k is not. The F protein is synthesized as a 68 kD precursor molecule (F0), which is enzymatically cleaved by a cellular protease into a disulfide-linked 48 kD (F1)and 20 kD (F2) heterodimer.

Figure 1. Schematic diagram of respiratory syncytial virus and respiratory syncytial virus genome. Genomic organization is depicted at the bottom of the figure with the 3' and 5' ends oriented to the left and and right respectively. Except for the nonstructural proteins 1B and 1C, other proteins are depicted by geometric shapes above the genome and their locations are indicated in the schematic drawing of the virion. Because the exact location of the 22k protein is not known, it is not depicted.

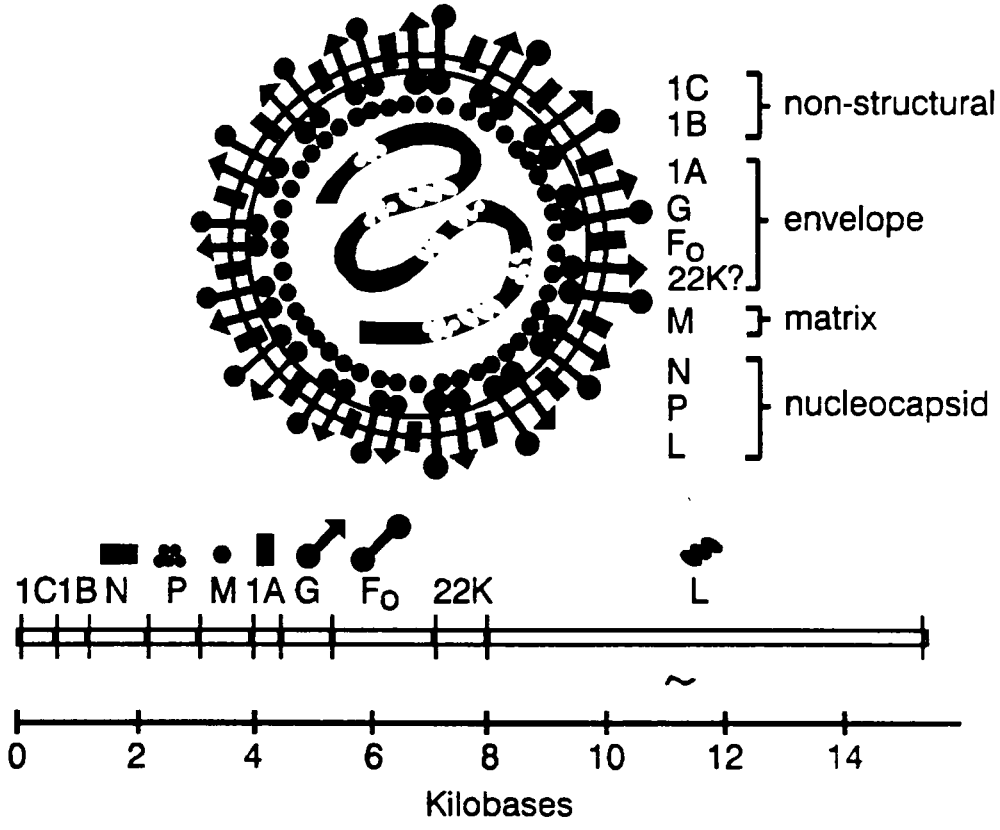
1C
1B
non-structural
1A
G
Fo
22K?
envelope
M
matrix
N
P
L
nucleocapsid
1C1B N P M 1A G Fo 22K
L
0 2 4 6 8 10 12 14
Kilobases

Table 1. RS virus genes: their products and functions (A2 strain of subgroup A)[a]

3'→ 5' order of RS virus genes	mRNA, # of nucleotides excluding poly (A)[b]	# of amino acids[b]	Protein Product: MW (kilodaltons): Unprocessed[b]	Protein Product: MW (kilodaltons): After glyco-sylation	Location	Function	Additional properties of gene product(s)
NS1 (1C)[c]	528	139	15.6	-	Not in virion	Not known	Slightly acidic
NS2 (1B)[c]	499	124	14.7	-	Not in virion	Not known	Basic
N	1197	391	43.5	-	Nucleocapsid	Structural protein of nucleocapsid	Abundant;tightly complexed with genomic RNA
P	907	241	27.1	-	Inner aspect of viral envelope	Component of polymerase complex?	Phosphorylated;relatively acidic;only one reading frame in distinction to the human parainfluenza virus type 3;no cysteine or tryptophane
M	952	256	28.7	-	Surface of infected cell	Inner lining of viral envelope	Relatively basic;moderately hydrophobic. Gene has second open-reading frame that partially overlaps COOH terminus of M coding region capable of coding for 75 amino acids; this product has not been identified
SH (1A)[c]	405	64	7.5	15 and 21-30	Surface of viral envelope	Not known	Strongly hydrophobic core of 28 amino acids. Inserted into outer membrane of infected cell with the C-terminal end extracellular
G	918	298	32.6	84-90	Surface of viral envelope	Attachment to host cell receptors	High content of carbohydrate, 90% of which is O-linked to serine and threonine. Protein has unusually high serine and threonine content (31%). Lacks NH_2-terminal signal sequence. Appears to be anchored in membrane by hydrophobic region (amino acids 38-66) near NH_2 terminus
F	1899	574	63.5	68-70	Viral envelope	Fusion of viral envelope with host-cell envelope; syncytium formation	NH_2-terminal signal peptide and COOH-terminal membrane anchorage domain. Cleaved proteolytically to yield disulfide linked F_2 (20 kd) and F_1 (48 kd). F_1 has an NH_2 terminal hydrophobic fusion domain. F_2 is NH_2 terminal with respect to F_1. F_2 is relatively hydrophilic and contains 4 of 5 sites for addition of N-linked carbohydrate side chains
M2 (22K)[c]	957	194	22.2	-	Nucleocapsid	Not known	Hydrophilic and very basic. Gene has second reading frame capable of reading frame just overlaps longer upstream reading frame. Putative second protein has not been identified
L	6578	2165	250	-		Polymerase of nucleocapsid?	Low abundance in virions and infected cells

[a]Adapted from McIntosh and Chanock, 1990.
[b]Deduced from nucleotide sequence of cDNA cloned from viral mRNA.
[c]Former designations.

After proteolytic cleavage, the F protein causes the virus envelope or membrane of virus-infected host cell to fuse with the membrane of uninfected cells and form syncytia (75). The G glycoprotein is necessary for virion attachment to host cells (71). Unlike the attachment proteins of the morbilliviruses and paramyxoviruses, the G glycoprotein lacks hemagglutinin or neuraminidase activities (130). The G glycoprotein is unique from other viral glycoproteins in that it is heavily glycosylated (primarily O-linked). The predicted molecular weight of the protein without glycosylation is approximately 32.6 kD, yet after the addition of the O-linked sugars, the molecular weight is approximately 84-90 kD (130). The 1A and 22k proteins are components of the virus envelope, but have unknown function (75). 1A, an integral membrane protein, is inserted in the outer membrane of the infected cell and its carboxy end is exposed to the extracellular environment (75). The 22k protein is not glycosylated and its exact location is unknown (75), but it may be a second matrix protein (M2) (75). The matrix (M) protein forms an inner lining of the virus envelope and is probably analogous to the matrix proteins of other enveloped RNA viruses (75). The functions of the nonstructural proteins 1B and 1C proteins are unknown (75).

Subgroup and Antigenic Characteristics

As RSV isolates have been characterized and compared antigenically and genetically, a basis for division of the pneumoviruses into subgroups has become evident. Two HRSV subgroups (A and B) were initially defined based on monoclonal antibody reactivity with certain structural proteins (3,81,91). This separation of HRSV strains is primarily based on major antigenic differences among

their G glycoproteins, however, minor differences also have been detected among F, N, M, and 22k proteins. These data suggest that HRSV subgroups evolved separately (81). Subgroup classification of BRSV, CRSV, and ORSV is less clear. Most monoclonal antibodies against structural proteins of HRSV subgroups A and B reacted with three BRSV strains and CRSV, however monoclonal antibodies against HRSV G glycoproteins did not react with BRSV or CRSV strains (91), suggesting that, although BRSV, CRSV, and HRSV strains share many epitopes, their G glycoproteins are antigenically distinct.

Based on immunodetection techniques with polyclonal antisera, the structural protein components of HRSV and BRSV are very similar (69,118). Only minor differences in molecular weight exist between corresponding proteins (19,69,118). Western blot and immunoprecipitation comparisons between BRSV and HRSV indicated that there was serologic cross-reactivity between their F, N, M, and P proteins while BRSV and HRSV G glycoproteins have major antigenic differences (69,118). Furthermore, while BRSV and HRSV mRNAs had similar electrophoretic mobilities, they cross hybridized poorly or not at all (69). In another study, HRSV, BRSV, and CRSV were compared by Western blot analysis; the HRSV and BRSV G glycoproteins only reacted with homologous antiserum, supporting the concept that these proteins were distinct (118). Anti-BRSV serum also reacted with the G glycoprotein of CRSV suggesting that BRSV and CRSV G glycoproteins are related (118).

More recently, G glycoprotein gene nucleotide sequences from HRSV A and B subgroups and BRSV strain 391-2 have been compared (68). Collectively, these data also support the existence of two HRSV subgroups and classification of BRSV in an additional RSV subgroup.

It is not clear whether BRSV strains constitute more than one antigenic type, because only few isolates have been analysed. Interestingly, according to Lerch et al, the G glycoprotein from BRSV strain 391-2 reacted with anti-BRSV strain 391-2 but not anti-BRSV strain 127 serum in Western blots, suggesting that two BRSV subgroups exist (69).

HRSV subgroups A and B cocirculate during respiratory disease outbreaks (45,80,102) and respiratory disease caused by these two subgroups may not differ clinically (43). However, some suggest that HRSV subgroup A infections tend to cause more severe disease (74). Prevalence of the HRSV subgroups in humans varies both during outbreaks and from outbreak to outbreak, but generally, researchers have demonstrated a predominance of HRSV subgroup A (43,80,102). There is no evidence that this variation allows reinfection to occur. Outbreaks of BRSV-associated disease vary in severity (55), however, the impact of more than one BRSV subgroup on clinical severity or pathogenesis of the disease remains to be determined.

Viral Target Antigens

Targets for Antibodies

Infection in humans with either subgroup of HRSV elicits cross-reactive and subgroup-specific antibodies against the F and G glycoproteins respectively (43). In another study, antibody reactivity in all age groups was directed against F, N, P, and M proteins, but antibody against the G glycoprotein was not detectable in patients less than one year old (96). In BRSV-infected calves, antibodies develop to the L, G, F, N, P, M, 22k, and 1A proteins (132). The antigenicity of the 1B and 1C proteins is unknown (55).

Immunization of cotton rats with vaccinia recombinant viruses and with purified proteins demonstrated that G and F proteins induce neutralizing antibodies that protect against challenge inoculation (15,55,86,89,122,123). In contrast, rats vaccinated with a vaccinia recombinant virus that expresses the N protein of HRSV were less protected than those immunized with vaccinia recombinants expressing the F or G glycoprotein (55). This suggests that F and G are the major target antigens necessary for protection. Mice and cotton rats immunized with a vaccinia recombinant expressing a G glycoprotein of a subgroup A HRSV were protected against challenge inoculation with a subgroup A HRSV virus, but not with a subgroup B HRSV virus (55). Those mice immunized with a vaccinia recombinant that expressed a subgroup A F protein were protected against challenge inoculation with virus of either HRSV subgroup (55). These observations indicate F protein from one HRSV subgroup elicits cross-protective antibodies against the other subgroup whereas G protein does not.

Four or five epitopes have been identified on the F protein and three or four of these sites are targets for neutralizing antibodies (55). Monoclonal antibodies directed against at least two of these epitopes inhibit fusion (55). The G glycoprotein of HRSV subgroup A has at least three antigenic sites, while the G glycoprotein of HRSV subgroup B has at least two (55). The interaction of antibodies with two of the subgroup A G glycoprotein antigenic sites and both of the subgroup B antigenic sites results in virus neutralization (55).

Targets for T Cells

The N and F proteins are the major antigens recognized by cytotoxic T cells in humans and mice and by helper T cells in mice based on studies using vaccinia

recombinant viruses expressing the G, N, and F proteins (55). The 1A protein is also recognized by helper T cells in mice (55).

Protective Immunity and Immunopathogenesis

Because HRSV infections are most common during the first six months of life when maternally transmitted serum antibody is almost universally present in children, concurrent presence of high serum antibodies and severe disease led to speculation that an immunopathogenic mechanism might be involved (75). Also, the adverse outcome of a clinical trial using a formalin-inactivated HRSV vaccine in children suggested that humoral immunity might predispose infected infants to the development of bronchiolitis (75). However, several observations tend to contradict this speculation. Recurrent HRSV infections occur readily, even in the face of passively or actively acquired immunity (55). Children may have severe disease after a second HRSV infection, but subsequent infections decrease in severity (55). This partial protection is somewhat greater against viruses of the same subgroup (55). Likewise, active immunity to BRSV protects calves from development of clinical disease, but not from reinfection (55). Bronchiolitis is most common in children six weeks to six months of age, but infants less than six weeks of age are relatively protected (75). Maternal antibody concentrations are highest at approximately three weeks of age and also are universally present in this age group (75). Some infants, without measurable neutralizing antibody, develop bronchiolitis suggesting a poor correlation between serum antibody levels in infants and disease severity (75). Finally, an association between low umbilical cord antibody concentration and the subsequent early development of serious lower respiratory

tract disease in children supports a protective rather than an immunopathogenic role of antibodies (75).

The Role of Antibodies

Laboratory animals have been used to study actively acquired immunity and the protective effect of serum antibodies in RSV infection. Immunization of cotton rats with HRSV F protein or a novel FG chimeric protein from a recombinant baculovirus stimulated serum neutralizing antibodies and protected rats from HRSV challenge (122,123). Purified F and G proteins administered to cotton rats were highly immunogenic and potentiation of pulmonary pathology did not occur after challenge inoculation (86). Vaccinia recombinant viruses expressing F or G proteins also induced high levels of specific serum neutralizing antibodies and almost complete resistance to RSV replication in the lower respiratory tract (55,89). Several immunopathogenic roles of antibodies have been proposed in RSV infection. Type I hypersensitivity may develop in children with HRSV infections (55,127). IgE was bound to exfoliated nasopharyngeal epithelial cells in infants and young children with acute RSV infection, especially in patients with bronchiolitis (126). RSV-specific IgE and histamine concentrations were elevated in respiratory secretions of patients with bronchiolitis and wheezing (129). Investigators have observed an association between early and high anti-HRSV IgE antibody levels in secretions and serum and the severity of lower respiratory tract disease (18,55,125,127). However, not all children hospitalized for a HRSV infection have detectable HRSV-specific IgE (55,127,128) particularly during the early stages of primary infection.

In cattle, anaphylaxis can be mediated by IgG1 and IgE (55). Although there was no clear correlation between BRSV-specific serum IgE concentration and clinical signs of disease following experimental BRSV infection (110), another study suggested that serum IgE concentrations are related to disease severity (109). Using a BRSV-specific enzyme-linked immunosorbent assay, Stewart and Gershwin showed that BRSV-specific IgE is often generated in calves in response to experimental BRSV infection and high serum IgE levels were correlated with clinical severity (109). They also showed a slight correlation between clinical severity of BRSV infection and lung lavage IgE content and a greater correlation between clinical severity and histamine content of nasopharyngeal exudate (110). They suggested that the data supports, at least indirectly, a role of IgE in the pathogenesis of BRSV related disease. IgE responses have not been examined in calves with severe natural disease.

The enhancement of severe disease by a formalin-inactivated vaccine used in children, also supports an immunopathogenic mechanism. Formalin may actually destroy epitopes that induce neutralizing and fusion-inhibiting antibodies on the G or F protein (55,85,87,99). Formalin-inactivated vaccine may induce predominantly "non-functional" antibodies that bind virus but can not neutralize infectivity or inhibit cell fusion. There is speculation that the presence of these non-neutralizing antibodies at the time of infection or their production at an accelerated rate after infection, may enhance disease by causing an Arthus reaction (type III hypersensitivity) (55). The F protein seems capable of inducing these non-functional antibodies (11,55,101). The role of these antibodies in complement activation was not studied. Some monoclonal antibodies directed against the F protein neutralize some strains of HRSV, but only bind to and fail to neutralize

other strains (11). A formalin-inactivated vaccine was administered to cotton rats and enhanced disease was observed after challenge inoculation (99). Additionally, histologic evidence of an Arthus reaction was observed 24 hours after challenge inoculation of the rats and an influx of neutrophils and lymphocytes four days after challenge, suggested that a delayed type hypersensitivity reaction (type IV hypersensitivity) may have developed.

Antibodies may form antigen-antibody complexes and induce injury during RSV illness. Even in the absence of antibody, HRSV- and BRSV-infected cells activate more complement than uninfected cells (31,55,108). Antibodies increase complement activation and cooperation of antibody and complement is required for induction of complement-mediated lysis (53,55). Complement activation was enhanced by BRSV-specific IgG1 and IgM, but not IgG2 or IgA and caused cell lysis (53,55). Murine monoclonal antibodies directed against neutralizing and non-neutralizing epitopes on the F protein also may enhance complement activation. One monoclonal of the IgG1 isotype directed against a non-neutralizing epitope on the F protein enhanced C3 binding to infected cells, but did not induce complement-mediated lysis (53,55). Thus, antibodies of the IgG1 and IgM isotypes directed against this epitope could conceivably participate in the activation of the complement cascade and mediate adverse inflammatory effects, and yet not induce a beneficial cell lysis or neutralize the virus (55).

An *in vivo* study of natural BRSV disease in cattle suggested a role for complement and the subsequent release of mast cell mediators in pathogenesis (54). In the lungs of calves naturally infected with BRSV both viral antigen and complement were present in only the cranioventral lung. However, reduced mast cell density, mast cell granules, and amount of histamine were present in both the

cranioventral and caudodorsal lung areas. This suggested that virus in the cranioventral lung activates complement resulting in subsequent mast cell activation and liberation of anaphylatoxins in all lung areas. This mechanism might explain the diffuse nature of the pulmonary lesion in BRSV-related disease, despite the cranioventral localization of the virus.

Antibody may also enhance RSV disease by facilitating viral infection of monocytes and macrophages. The role of the mononuclear phagocyte system in RSV infection is unclear due to conflicting data regarding the permissiveness of cells of monocyte/macrophage origin to RSV infection. Human alveolar macrophages, monocytes, and macrophages as well as cell lines of macrophage lineage are generally permissive to RSV infection (29,39,62,78,93) particularly in the presence of RSV-specific antibodies (39,62). The exact mechanism of enhancement is not clear, but non-neutralizing or subneutralizing concentrations of antibody may complex with virus particles, promote contact of complexed virus with the cell surface Fc receptors, and facilitate entry of the virus into the cell (62). When cells bearing Fc receptors become infected or interact with the virus, they may release leukotrienes and platelet-activating factor, which could cause bronchoconstriction, as often seen in the disease (55).

Trigo et al examined the effect of bovine respiratory syncytial virus on selected functions of bovine pulmonary alveolar macrophages and observed abortive infections when bovine pulmonary alveolar macrophages were infected with BRSV (115). This suggested that bovine alveolar macrophages may not be totally permissive to BRSV infection. The ability of BRSV-infected alveolar macrophages to phagocytize killed Staphylococcus epidermidis or latex beads was similar to uninfected alveolar macrophages, however, infected macrophages had significantly

impaired phagocytosis of antibody-coated sheep red blood cells in spite of normal Fc receptor function. The authors suggested that BRSV infection of alveolar macrophages produces defective Fc receptor-mediated phagocytosis, but not generalized depression of the phagocytic process.

The Role of Maternal Antibodies

In both human infants and calves, anti-RSV maternal antibodies are common, as a result of frequent recurrent RSV infections in the mothers and dams (55). Anti-HRSV maternal antibodies in children may be exclusively IgG1 (46) and are usually directed against the F and G proteins (70,82,83). In calves, the passively acquired anti-BRSV antibodies consist of IgG1 too and are directed against the F and N proteins, although some calves also have maternal antibodies against the G protein (55,56,58,132). Calves, in contrast to humans, acquire maternal antibodies only by ingestion of colostrum (55).

While a humoral immunopathogenic mechanism has been proposed in the pathogenesis of RSV infection, evidence exists that maternal antibody may provide some, yet incomplete protection. Maternal antibody reduced virus shedding *in vivo* and there was a correlation between the level of neutralizing maternal antibody at birth and the age at the time of infection (21,40,41,55,63,88,121). In hospitalized infants, the severity of disease peaks in the second month of life, when maternal antibodies are still present (55,94). However, no correlation exists between serum antibody level and disease severity (16,55,94). In addition, infants less than three weeks old, who have the highest concentration of maternal antibodies, are relatively spared from severe disease (55). Similarly, during the seasonal circulation of BRSV

among cattle, disease can frequently be observed in calves two weeks old and older. However, most cases of severe disease occur in calves one to three months old and nearly all of these calves have maternal antibody (55,59). The incidence and severity of disease in calves younger than three months of age are inversely related to the level of maternal antibody. Therefore, while maternal antibodies do not appear to prevent BRSV-associated disease, they may abrogate it (55,59).

Passive transfer of anti-HRSV antibodies to laboratory animals confers protection without exacerbation of disease. Passive transfer of monoclonal antibodies directed against F and G proteins reduced HRSV replication in the lungs of mice and cotton rats (55,113,120). In cotton rats, passively-transferred serum from HRSV-infected rats almost completely inhibited HRSV replication in the lung, but viral shedding from the nose was only slightly affected (97,98). Intravascular administration of human hyperimmune serum with high concentrations of anti-HRSV neutralizing antibodies significantly reduced the amount of HRSV recovered from the lung and the nose of owl monkeys challenge inoculated with HRSV (44). Passive immunity derived from colostrum, also decreased the severity of BRSV infections in calves (12). These observations support a protective role for maternal antibodies rather than an immunopathogenic one.

Some studies suggested that maternal anti-RSV antibodies in infants and calves suppressed active serum and mucosal antibody responses of all isotypes, despite extensive replication of the virus. Anti-HRSV maternal antibodies may cause poor immunological responses against the F and G proteins, even in children as old as eight months (55,82). In calves, the IgM response may be the least sensitive to suppression (55,56,131). Although antibody response to the whole virus is strongly inhibited by maternal antibodies, responses to certain viral proteins,

especially the F and P proteins can sometimes be detected (55,57,70,132). In cotton rats, antibody responses against vaccinia recombinant viruses expressing the F and G proteins were suppressed by passively transferred HRSV immune serum, but antibody responses against the vaccinia virus antigens were not (55,84).

The ratio between antibodies directed against protective and non-protective epitopes may be an important determinant of disease severity (55). In cotton rats, the antibody response to neutralizing epitopes was suppressed more than the response to nonneutralizing epitopes on the same protein by passively transferred hyperimmune serum (55,84). Cotton rats, whose immune responses were suppressed by passively transferred antibodies, were more susceptible to HRSV infection than control animals (55,84).

Maternal antibodies may also suppress T cell responses. Bangham demonstrated that passively acquired antibody in newborn mice impaired the antibody response and generation of specific cytotoxic T cell precursors (9,55).

The Role of Mucosal Antibodies

Mucosal IgA may be beneficial in HRSV infections of humans because the appearance of specific IgA coincides with elimination of the virus during recovery from initial HRSV infection (76,77). In human adults, resistance to HRSV infection and illness correlated with high levels of neutralizing antibody in the nasal cavity at the time of infection, but not with the level of neutralizing antibody in the serum (79). However, children that lack a secretory antibody response because of suppressive effects of maternal antibody, can normally recover from HRSV infection and similarly, calves with suppressed IgA responses due to maternal

antibodies did not shed virus any longer than calves without maternal antibodies (56,76).

Eight to ten days after primary BRSV infection of calves, BRSV-specific IgM was detected in serum and samples collected from the eye, nose, lungs, and the intestine and shortly afterward IgA was detected (56). BRSV-specific IgG1 and IgG2 was detected later, only in the serum. In calves with maternal immunity, antibody responses were either undetectable or detectable only for short periods and at low titer. All calves, in this study, shed similar amounts of virus for similar lengths of time regardless of the level of maternal immunity. When these calves were reinfected three to four months later, BRSV-specific memory antibody responses were detected in serum and at the mucosae in all calves regardless of previous maternal immunity. Memory antibody responses were characterized by rapid increases in mucosal IgA and IgM and serum IgA, IgG1, and IgG2. Memory antibody responses were even detected in calves that did not develop a primary antibody response. Maternal antibodies at the time of priming, adversely affected the maximum antibody titers after challenge inoculation, either because priming was inefficient or because the immune response continued to be suppressed. After reinfection, none of the calves excreted virus, regardless of the presence of maternal antibodies at the time of priming. These data suggest that local challenge inoculation of the respiratory system in calves primes for local and systemic antibody memory responses and are associated with protection against virus excretion even if calves possess maternal antibodies.

The Role of T Cells

Little is known about the role of T cells in the recovery from and protection against RSV infection. Proliferative T cell responses have been detected after infection of calves and infants (25,103-105,112). Virus-specific MHC-restricted cytotoxic T cells have been detected in mice, cotton rats, and humans (10,55,90). Virus-specific helper T cells have also been detected in mice (55). Non MHC-restricted natural cytotoxity has been detected in cotton rats (55).

T cells may provide protection from RSV infection. Virus-specific lymphocyte transformation occurred in calves that were experimentally infected with BRSV (112). Calves that were vaccinated with an inactivated vaccine developed a virus-specific lymphocyte transformation response that was related to increased resistance to BRSV infection and not exacerbation of BRSV related disease following experimental inoculation (112). Infants and mice with defective cell-mediated immune response are unable to eliminate a HRSV infection further supporting a beneficial role of T cells (55). Furthermore, the transfer of RSV-primed T cells cleared persistent HRSV infection in immunodeficient mice (55). However, transfer of HRSV-specific cytotoxic T cells into infected mice resulted in clearance of virus, but also was associated with fatal respiratory disease characterized by pulmonary hemorrhage and neutrophil influx suggesting that cytotoxic lymphocytes may play a role in RSV disease pathogenesis (55,75).

The pathogenic role of cell-mediated immunity was also suggested by increased lymphocyte transformation responses in infants, previously vaccinated with an inactivated HRSV vaccine, with severe RSV infections (105). Some researchers have found significantly higher HRSV-specific lymphocyte

transformation responses in hospitalized infants less than six months old as compared to older infants (104). This observation temporally associates heightened lymphocyte transformation responses to the age group that typically experiences the most severe RSV infections. Contradictory observations of other researchers have indicated that among patients with RSV infection, the lymphocyte reactivity to RSV occurred significantly less often in babies six months of age or less than in patients over six months of age (103). RSV-specific lymphocyte reactivity peaked usually within one week after infection and appeared to be age dependent with 65% of infants 6-24 months of age and only 35-38% of infants under five months of age exhibiting cellular cytotoxicity to RSV (22). Conflicting evidence has left the role of the cell mediated immunity in RSV pathogenesis unclear.

CHAPTER 2

INTRODUCTION AND JUSTIFICATION

The G glycoprotein of respiratory syncytial virus (RSV) is unique among viral proteins. It has an approximate mature molecular weight of 84-90 kD, yet interestingly, cDNA clones prepared from HRSV mRNA encode a protein of only 32.6 kD (130). This suggests that over 50% of the molecular weight of the mature glycoprotein may be contributed by carbohydrate moieties (130). Resistance to tunicamycin inhibition of N-linked glycosylation suggests that most carbohydrate moieties are primarily linked by O-glycosidic bonds (130). Glycoprotein G nucleotide gene sequences encode a high percentage of serine and threonine residues, the potential acceptor sites for O-linked oligosaccharides (130). Amino acid sequences, deduced from G glycoprotein gene sequences, lack amino terminal signal sequences and carboxy terminal hydrophobic sequences that might serve as a membrane spanning domain (130). However, a strongly hydrophobic region located near the amino terminus may serve both as the signal sequence and the membrane spanning domain of the protein (130). The protein is likely oriented in the membrane with the amino and carboxy termini forming the cytoplasmic and extracellular domains respectively (68).

The G glycoprotein functions as a factor for virion attachment to host cells (71). Prototypical paramyxoviruses have two major membrane glycoproteins, F which is responsible for cell fusion and HN or H which is responsible for neuraminidase and/or hemagglutinating activities (130). Respiratory syncytial virus has a fusion (F) glycoprotein, but the G glycoprotein has neither hemagglutinating or neuraminidase activities and there is neither structural nor sequence homology between G and HN or H (36).

Human and bovine RSV G glycoprotein mRNAs and genes have been characterized primarily by cDNA cloning and nucleotide sequencing. To date published sequence data from HRSV subgroups A and B and a BRSV isolate have been reported (52,68). Lerch et al reported the sequence of the G glycoprotein BRSV strain 391-2 and compared nucleotide and amino acid identities between BRSV strain 391-2 and G glycoproteins genes from HRSV strains A2 and 18537 (68). The BRSV strain 391-2 G glycoprotein gene nucleotide sequence and deduced amino acid sequence shared 51% nucleic acid identity and 30% amino acid identity, respectively, with the HRSV counterparts (68). The nucleic acid identity and the amino acid identity between HRSV subgroups was 67.4% and 53%, respectively (68). Amino acid identities among G glycoproteins from strains within the same HRSV subgroup is approximately 94% (52). These data are consistent with the existence of two HRSV subgroups and a separate BRSV subgroup. HRSV G glycoprotein amino acid sequences, regardless of subgroup, have a conserved region of 13 amino acids in the proposed extracellular domain. The latter may be the receptor binding site (68). Only six of the thirteen amino acids of the BRSV strain 391-2 G glycoprotein are conserved with the HRSV counterparts, yet four cysteine residues, two within this region and two immediately adjacent to this region are conserved exactly between the BRSV and HRSV G glycoproteins (68). These conserved cysteine residues could confer similar secondary structure among the G glycoproteins, but amino acid differences within the conserved 13 amino acid region could be responsible for host range specificity (68).

The G glycoprotein is important for the induction of a neutralizing and protective immune response (55). This is best demonstrated by experimental observations of protection of experimental animals against RSV challenge after

passive transfer of monoclonal antibodies against G glycoprotein, immunization with purified antigen, or inoculation of vaccinia recombinants expressing the G glycoprotein (previously discussed). Researchers have clearly demonstrated with vaccinia recombinant viruses expressing HRSV G glycoprotein, the RSV immune response induced, protects animals only against respiratory syncytial viruses of the same subgroup (55).

HRSV subgroup divergence could be important in HRSV infections and epidemiology. Evidence exists that BRSV may also have divergent subgroups (69). This could have tremendous implications in future investigations. With two BRSV subgroups, a bovine model of the human disease could establish the importance of subgroup differences in immunity and immunoprophylaxis. This model may have advantages over others used in the past in that BRSV could be used in its natural host and the disease in calves and human infants is very similar. Unfortunately, except for BRSV strain 391-2 (68,69), other BRSV strains and two small ruminant RSVs, CRSV and ORSV, have not been well characterized. CRSV and ORSV affect two additional ruminant species (33,64,107) and can also infect other ruminant species as well (17). Molecular characterization of their G glycoprotein genes may not only be valuable in establishing the host range differences of each virus, but these genes may also prove useful in future vaccine development.

For these reasons, we have begun to examine antigenic and genetic heterogeneity of various RSV isolates including CRSV, ORSV, two HRSV, and seven BRSV strains. The following pages include comparison studies of these various strains using many biotechniques including Western blot analysis, polymerase chain reaction (PCR), molecular cloning, dideoxy nucleotide chain termination DNA sequencing, and RNase A mismatch cleavage analysis.

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PART 2

COMPARATIVE ANALYSIS OF RESPIRATORY SYNCYTIAL VIRUS G GLYCOPROTEINS BY WESTERN BLOT ANALYSIS

CHAPTER 1

ABSTRACT

The existence of more than one subgroup of bovine respiratory syncytial virus (BRSV) may have important implications in disease pathogenesis and immunoprophylaxis. The purpose of this study was to compare immunoreactivities of G glycoproteins from various RSV strains by a polyclonal serum immunodetection system. We used seven bovine (375, 391-2, FS1, NMK7, 1143, 1144, and 16186) one caprine (CRSV), one ovine (ORSV), and two human strains (A2 and B8/60) of respiratory syncytial virus (RSV) and compared immunorecognition of the G glycoprotein of each by Western blot analysis with two polyclonal sera. Gnotobiotic bovine anti-BRSV strain 375 serum reacted with the G glycoprotein of all bovine strains, but did not react with the G glycoprotein of CRSV, ORSV, or HRSV. Human anti-HRSV serum reacted with the G glycoprotein of human A2 strain only. The data is in agreement with previously published data that suggests BRSV strains represent a third RSV subgroup. CRSV and ORSV strains may represent an additional subgroup(s). A previous report suggested the existence of at least two BRSV subgroups detected by Western blot analysis with polyclonal sera. Glycoprotein G heterogeneity was not detected by polyclonal immunoelectrophoresis among all BRSV strains tested.

CHAPTER 2

INTRODUCTION

Respiratory syncytial virus (RSV), an important cause of serious lower respiratory tract disease, causes similar disease in both infants and calves (7). Both bovine respiratory syncytial virus (BRSV) and human respiratory syncytial virus (HRSV) are classified in the pneumovirus genus in the family Paramyxoviridae (11); produce similar clinical disease predominantly in neonates (7); occur regardless of maternally-derived, passively acquired immunity (7); and produce similar pathologic lesions (2). Repeated infections with RSV occur in both humans and cattle although subsequent infections are less severe (7).

Respiratory syncytial virus is an enveloped virus with a negative sense, single stranded RNA genome (4). The genome has ten genes spread over approximately 15,000 nucleotides; each encodes a unique mRNA (4) transcribed from a single promoter at the 3' end (5). The mRNAs are translated into ten unique proteins, comprising eight structural and two nonstructural proteins (11). Of these eight structural proteins, two glycoproteins (G and F) constitute the major surface antigens of infected cells and virions (11). Both of these surface glycoproteins have been shown to be important immunogenic proteins (6,13,16-18). Most of the molecular characterization of RSV has come from studies of HRSV, however, recently the proteins and mRNAs of BRSV strain 391-2 have been characterized and cDNAs derived from viral mRNAs have been cloned (10).

Human respiratory syncytial virus antigenic variation has been characterized by immunoreactivity of viral proteins with panels of monoclonal antibodies (1,12),

providing a basis for separation of HRSV strains into either subgroup A or B. Variable epitope differences were found in four structural proteins: G, F, M, and N, but the only major differences were detected in the G glycoprotein (12). A similar comparison of HRSV, BRSV, and CRSV strains also demonstrated major antigenic differences between the G glycoproteins of the two HRSV subgroups (14). Additionally, monoclonal antibodies against the G glycoprotein of either HRSV subgroup did not react with BRSV or CRSV strains (14), suggesting that BRSV and CRSV belonged to an additional subgroup(s). Most but not all monoclonal antibodies against the other structural proteins of HRSV subgroup B cross-reacted with proteins of both HRSV subgroups, BRSV, and CRSV (14). Other investigators have compared the structural proteins of HRSV, BRSV, and CRSV (3,10,15), verifying that distinct antigenic differences exist between G glycoproteins from HRSV and BRSV (10,15). Serologic cross-reactivity of G glycoproteins from BRSV and CRSV strains was detected (15). These studies collectively suggest that, in addition to the two HRSV subgroups, BRSV represents a distinct RSV subgroup that may be antigenically related to the CRSV.

Lerch et al have cloned cDNA from the G glycoprotein gene of BRSV strain 391-2 and compared its sequence to published sequence data from HRSV strains representing A and B subgroups (9). The BRSV G glycoprotein gene shares approximately 51% nucleic acid identity and 30% amino acid identity with the nucleotide and amino acid sequences of HRSV strains A2 and 18537 (9). Clearly, molecular differences at the genetic level warrant the classification of BRSV as a distinct RSV subgroup.

Multiple BRSV subgroups have not been identified with certainty because few bovine isolates have been characterized. However, one study demonstrated

immunorecognition of BRSV strain 391-2 G glycoprotein by anti-BRSV strain 391-2 serum, but not by anti-BRSV strain 127 serum, suggesting that BRSV also has at least two antigenic subgroups (10).

In this study, we used a Western blot procedure to characterize the G glycoprotein antigenic relatedness of BRSV strains from various geographic sources including Japan, North Carolina, Iowa, Colorado, and Texas. We also compared the antigenic relatedness of BRSV, HRSV subgroups A and B, CRSV, and ORSV G glycoproteins with polyclonal antisera against BRSV strain 375 and HRSV subgroup A.

CHAPTER 3

MATERIALS AND METHODS

Viruses and Cells

Eleven strains of RSV were obtained from various sources. HRSV strains A2 and B8/60 and BRSV strain 391-2 were provided by Dr. Gail Wertz (Department of Microbiology, The University of Alabama at Birmingham Medical School, Birmingham, AL). ORSV, CRSV, and BRSV strains 375, FS1-1, and NMK7 were provided by Dr. Howard Lehmkuhl (National Animal Disease Laboratory, Ames, IA). BRSV strains 1143, 1144, and 16186 were provided by Dr. James Collins (Colorado State University, Fort Collins, CO). Bovine turbinate cells (BTU-5) were provided by Dr. Kenny Brock (The Ohio Agricultural Research and Development Center, Wooster, OH). Viruses were grown in BTU-5 cells maintained in Dulbecco's Modified Eagle Medium (DMEM)(Hazelton Biologics, Inc., Lenexa, KS) and supplemented with 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD). When approximately 50% of the cells in the monolayer had formed syncytia, they were suspended by scraping into the medium and dimethylsulfoxide (DMSO) was added to equal 5% of the total volume as a cryopreservative. One ml aliquots were stored at -70 C. Respiratory syncytial virus infection of BTU-5 cells was confirmed by direct immunofluorescence with a fluoroscein-labelled anti-RSV fusion (F) protein monoclonal antibody (Baxter Healthcare Corp., Bellevue, WA).

Antisera

Bovine anti-BRSV strain 375 serum derived from a gnotobiotic calf was provided by Dr. Howard Lehmkuhl (National Animal Disease Laboratory, Ames, IA). Human anti-HRSV serum was provided by Dr. Dianne Murphy (The University of Tennessee Medical Center, Knoxville, TN).

Viral Protein Lysates

Confluent BTU-5 cell monolayers were infected with the various RSV strains. When approximately 50% of cells in a monolayer had formed multinucleated syncytia (4-6 days post-inoculation), proteins were harvested using a slight modification of a method described by Lerch et al (10). Infected cell monolayers were washed once with phosphate buffered saline (PBS) and drained well. One ml of lysis buffer (1% vol/vol Nonidet P-40; 0.4% wt/vol deoxycholic acid; 66 mM ethylenediaminetetraacetic acid (EDTA); and 10 mM Tris-HCl, pH 7.4) (Sigma Chemical Co., St. Louis, MO) was added to the monolayers. Following gentle rocking at 4 C for 20 minutes, the flasks were scraped and the contents were transferred to a 1.7 ml microfuge tube and the nuclei were removed by centrifugation at 10,000 rpm for 10 minutes. Protein lysates of each RSV strain plus uninfected BTU-5 cells were mixed with an equal volume of treatment buffer (Tris-HCl, 0.125 M (pH 6.8), 4% sodium dodecylsulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol) (Sigma Chemical Co., St. Louis, MO) and 0.05% volume of 1% bromphenol blue (in distilled water) (Allied Chemical Corp., New York City, NY) solution. Samples were placed in a boiling water bath for 90

seconds and immediately transferred to cool on ice before loading onto the gel apparatus or stored at -20 C for later use.

Polyacrylamide Gel Electrophoresis

Following a slight modification of a technique described by Laemmli (8), viral proteins were resolved by polyacrylamide gel electrophoresis with an SE 400 Sturdier Slab Gel Electrophoresis Unit (Hoefer Scientific Instruments, San Francisco, CA) according to manufacturer's instructions. The resolving gel consisted of 10% polyacrylamide, 0.375 M Tris-HCl (pH 8.8), 0.1% SDS, 0.1% w/v ammonium persulfate, 0.07% v/v N,N,N',N'-tetramethylethylenediamine (TEMED). The stacking gel consisted of 4% polyacrylamide; 0.125 M Tris-HCl (pH 6.8), 0.1% SDS, 0.05% w/v ammonium persulfate; and 0.05% v/v TEMED. The electrophoresis buffer consisted of 0.025 M Tris-HCl, pH 8.3, 0.192 M glycine; 0.1% SDS.

Forty μl of each lysate/treatment buffer mixture was loaded per lane and the proteins were electrophoretically separated at a constant current of 30 mA until the tracking dye front reached the bottom of the gel. One lane on each gel was loaded with pre-stained protein standards of known molecular weight (Bio-Rad Laboratories, Richmond, CA).

Electroblotting of Proteins

Proteins were electrophoretically transferred from the polyacrylamide gel onto Transphor (0.45 μm pore size) nitrocellulose (Hoefer Scientific Instruments,

San Francisco, CA) with a Biotrans semi-dry electrophoretic transfer unit (Gelman Sciences, Ann Arbor, MI) according to the manufacturer's instructions. Absorbent blotting paper and nitrocellulose was cut to the size of the polyacrylamide gel and soaked in transfer buffer (48 mM Tris base, 39 mM glycine hydrochloride, 1.3 mM SDS, and 20% methanol). Proteins were electrophoretically transferred at a constant current of 0.8 mA/cm^2 of membrane for one hour.

Detection of Blotted Antigens

A slight modification of a method described in Promega's Protocols and Applications Guide was used for detection of blotted antigens (Promega, Madison, WI).

Blotted membranes were rinsed in Tris buffered-saline plus Tween 20 (TBST) (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO)). Following one rinse the membrane was incubated in TBST with 1% blot qualified bovine serum albumin (BSA) (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and 1% normal rabbit serum at room temperature for 30 minutes to block excess protein binding sites on the nitrocellulose. Duplicate blots were treated with either bovine anti-BRSV strain 375 or human anti-HRSV antisera. After removal of the blocking solution from the membranes, primary antibody at a 1:100 dilution in TBST was added and the membranes were incubated at room temperture for 30 minutes. The membranes then were washed in TBST three times for five minutes to remove unbound antibody, followed by a 30 minute incubation at room temperature with enzyme-conjugated secondary antibody (rabbit anti-bovine IgG/alkaline phosphatase

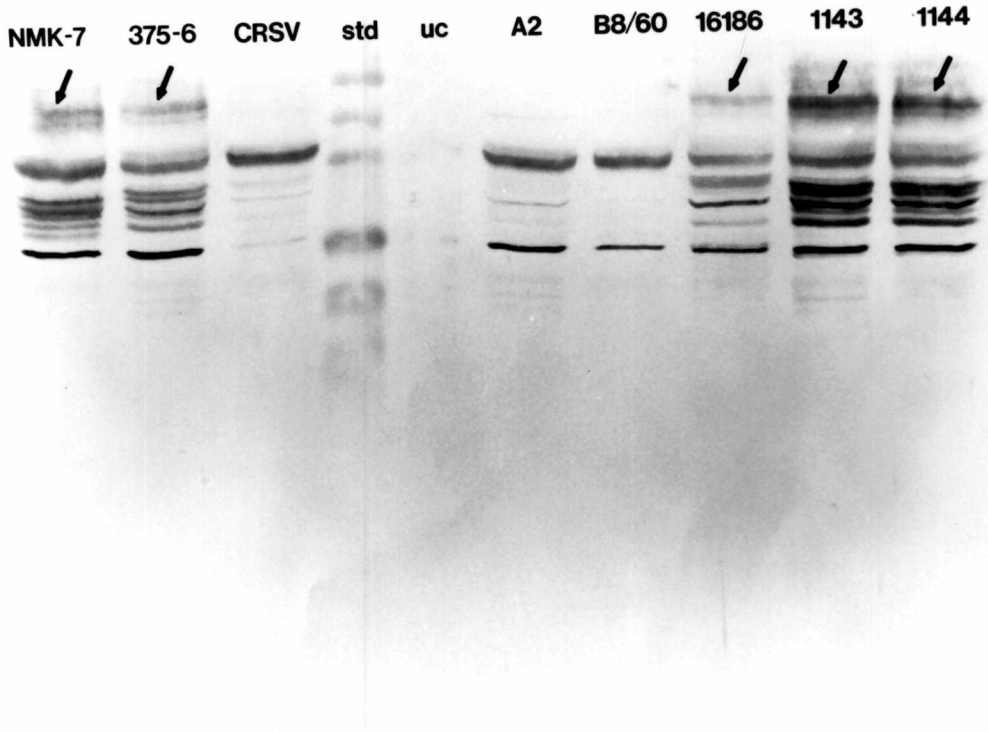
conjugate, Sigma Chemical Co., St. Louis, MO) at a dilution of 1:1000 in TBST. The membranes then were washed in TBST three times for five minutes to remove unbound antibody and conjugate. Membranes were blotted on filter paper and transferred to color development solution consisting of alkaline phosphatase (AP) buffer (100 mM Tris-HCl, pH 9.5; 100 mM NaCl; 5 mM $MgCl_2$), nitroblue tetrazolium (NBT) (66 μl/10 ml AP buffer), and 5-bromo, 4-chloro, 3-indolyl phosphate (BCIP) (33 μl/10 ml AP buffer). NBT (75 mg/ml) and BCIP (50 mg/ml) were supplied in dimethylformamide buffer from GIBCO BRL, Gaithersburg, MD. Incubations times were five to fifteen minutes. Color development was stopped by rinsing membranes in deionized water.

CHAPTER 4

RESULTS

In each lane of our immunoblots, we observed multiple protein bands with electrophoretic mobilities corresponding to molecular weights ranging from approximately 27-140 kD regardless of the antiserum used (Figures 1-4). In Figures 1 and 3, bovine anti-BRSV strain 375 serum reacted with a protein with an electrophoretic mobility of 85-90 kD in the lanes corresponding to all of the various bovine strains (375, 391-2, FS1, NMK7, 1143, 1144, 16186), whereas proteins with identical mobilities were not evident in the HRSV A2, HRSV B8/60, CRSV, or ORSV lanes. In lanes corresponding to the BRSV strains, 6-8 additional proteins with mobilities corresponding to molecular weights ranging from 27-50 kD reacted with the anti-BRSV serum, but in HRSV A2, HRSV B8/60, CRSV, or ORSV lanes, only 2 proteins cross-reacted consistently. These two proteins had mobilities corresponding to approximately 42 and 50 kD. In contrast, only the HRSV A2 lanes had a reactive protein with a mobility corresponding to a molecular weight of 85-90 kD when duplicate immunoblots were treated with the human anti-HRSV serum (Figures 2 and 4). However, a 85-90 kD protein was not evident in any BRSV lane or HRSV B8/60, CRSV and ORSV lanes (Figures 2 and 4). Common to all lanes, was a cross-reactive protein with an approximate molecular weight of 50 kD. No protein bands were observed in the lane containing uninfected BTU-5 cell protein lysate.

Figure 1. Western blot analysis of RSV strains NMK-7, 375-6, CRSV, A2, B8/60, 16186, 1143, and 1144 (treated with bovine anti-BRSV strain 375 serum). The BRSV G glycoproteins (85-90 kD) are indicated by arrows. Abbreviations include bovine RSV strains (375-6, NMK-7, 1143, 1144, and 16186); human RSV strains (A2 and B8/60); caprine RSV (CRSV); and uninfected cell total protein extract (uc). Molecular weight standards (std) from the top are 130, 75, 50, 39, 27, and 17 kD, respectively.


NMK-7
375-6
CRSV
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A2
B8/60
16186
1143
1144

Figure 2. Western blot analysis of RSV strains NMK-7, 375-6, CRSV, A2, B8/60, 16186, 1143, and 1144 (treated with human anti-HRSV serum). The HRSV strain A2 G glycoprotein (85-90 kD) is indicated by the arrow. Abbreviations include bovine RSV strains (375-6, NMK-7, 1143, 1144, and 16186); human RSV strains (A2 and B8/60); caprine RSV (CRSV); and uninfected cell total protein extract (uc). Molecular weight standards (std) from the top are 130, 75, 50, 39, 27, and 17 kD, respectively.

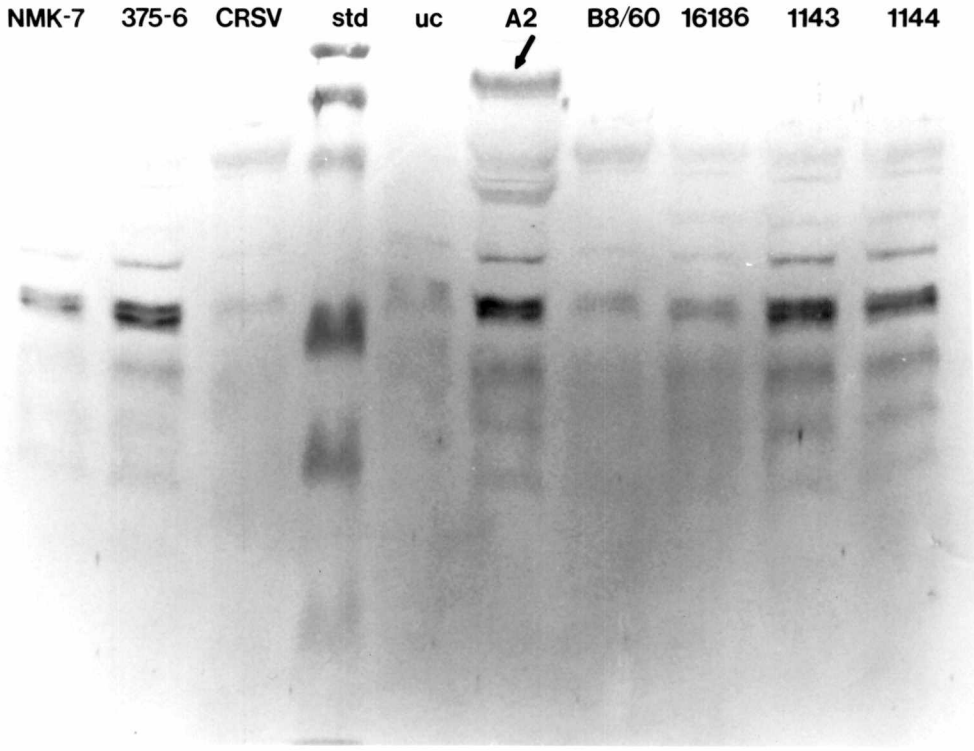

NMK-7
375-6
CRSV
std
uc
A2
B8/60
16186
1143
1144

Figure 3. Western blot analysis of RSV strains A2, B8/60, CRSV, ORSV, 375-6, 391-2, 16186, FS1-1, and NMK-7 (treated with bovine anti-BRSV strain 375 serum). The BRSV G glycoproteins (85-90 kD) are indicated by arrows. Abbreviations include bovine RSV strains (375-6, 391-2, FS1-1, NMK-7, and 16186); human RSV strains (A2 and B8/60); caprine RSV (CRSV); and ovine RSV (ORSV). Molecular weight standards (std) from the top are 130, 75, 50, 39, 27, and 17 kD, respectively.

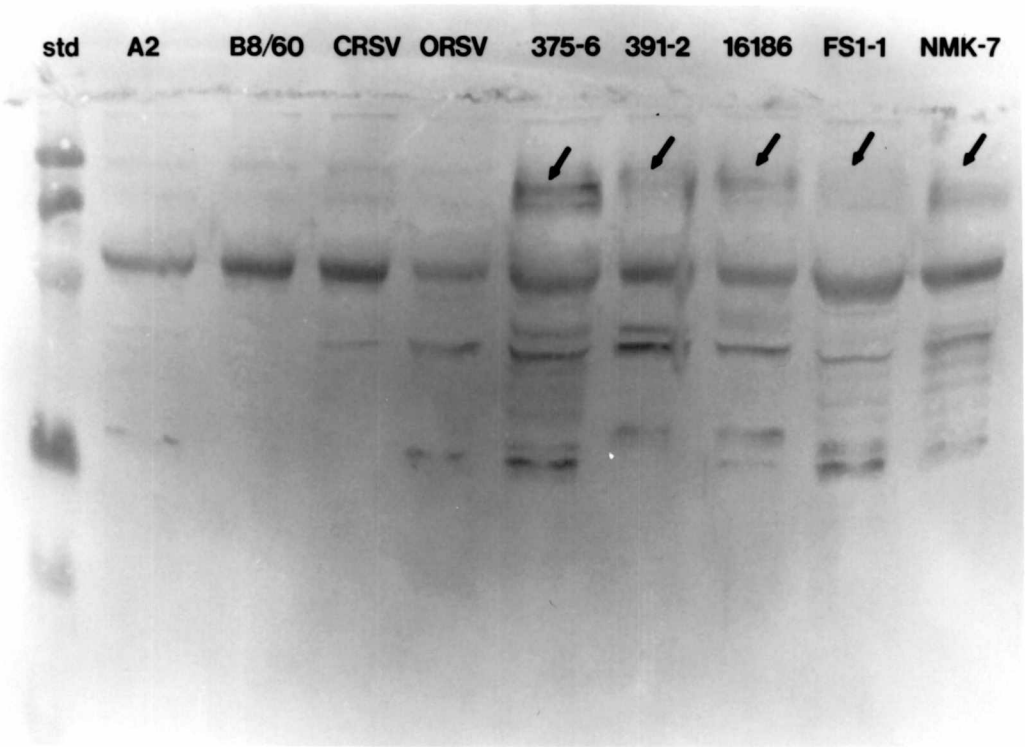
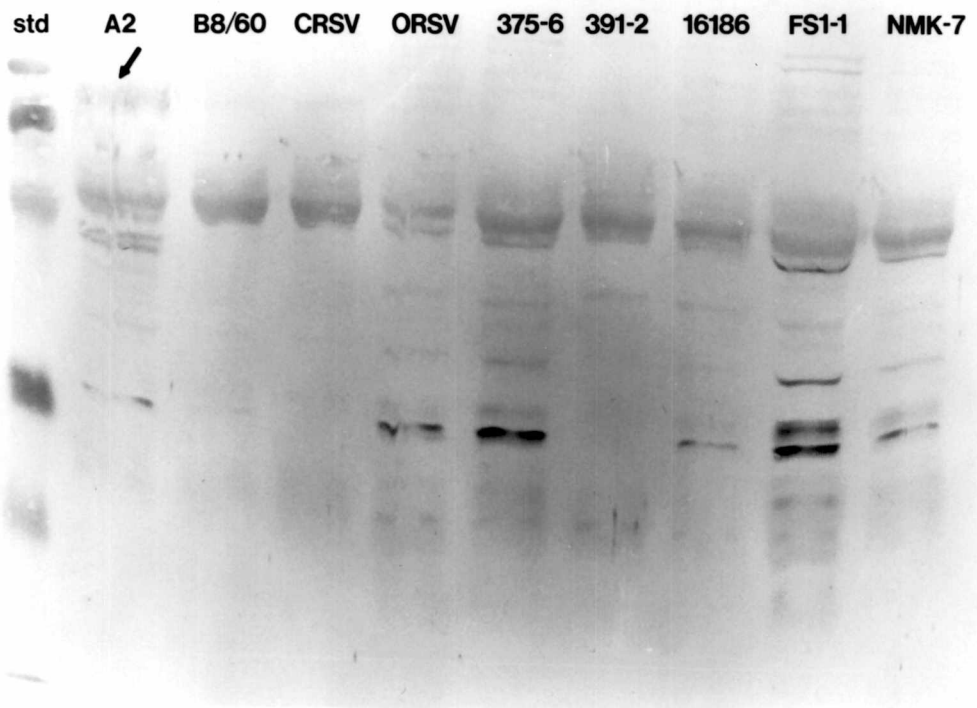

std
A2
B8/60
CRSV
ORSV
375-6
391-2
16186
FS1-1
NMK-7

Figure 4. Western blot analysis of RSV strains A2, B8/60, CRSV, ORSV, 375-6, 391-2, 16186, FS1-1, and NMK-7 (treated with human anti-HRSV serum). The HRSV strain A2 G glycoprotein (85-90 kD) are indicated by arrows. Abbreviations include bovine RSV strains (375-6, 391-2, FS1-1, NMK-7, and 16186); human RSV strains (A2 and B8/60); caprine RSV (CRSV); and ovine RSV (ORSV). Molecular weight standards (std) from the top are 130, 75, 50, 39, 27, and 17 kD, respectively.


std
A2
B8/60
CRSV
ORSV
375-6
391-2
16186
FS1-1
NMK-7

CHAPTER 5

DISCUSSION

We elected to include two RSV-specific antisera (anti-HRSV and anti-BRSV) in our study based on previous reports that clearly show distinct antigenic differences between the G glycoproteins of HRSV and BRSV strains (10, 15). Western blotting analysis allowed recognition of the RSV G glycoproteins of homologous and heterologous BRSV strains with anti-BRSV strain 375 serum suggesting that the BRSV strains included in our study, isolated from a variety of geographic sources, were antigenically related and likely should be classified in the same subgroup. Anti-BRSV strain 375 serum did not react with the G glycoproteins of CRSV, ORSV, or HRSV strains A2 and B8/60. Anti-HRSV serum reacted with the G glycoprotein of HRSV strain A2 only, but did not react with the G glycoprotein of HRSV strain B8/60, suggesting that the human antiserum was specific for the subgroup A G glycoprotein. Also, the human anti-HRSV serum did not react with G glycoproteins of CRSV, ORSV, or any BRSV strain suggesting that the A2 G glycoprotein is antigenically different from G glycoproteins of the bovine and small ruminant strains. Because the G glycoproteins of CRSV and ORSV did not react with either antiserum, we concluded that their G glycoproteins were antigenically distinct from the human and bovine subgroups, however, we did not conclusively distinguish CRSV and ORSV from HRSV strain B8/60. Most RSV strains tested had cross-reactive proteins of approximately 50 and 42 kD. These are likely the F_1 and N proteins. Additional proteins were noted both in BRSV and HRSV lanes. We considered these likely to be non-specifically reactive proteins. We did not pre-adsorb our antisera to minimize reactivity with cell culture proteins

before use in our Western blot analyses. However, minimal cross-reactive proteins were evident in the uninfected cell control lane. Reactivity of the bovine anti-BRSV strain 375 serum with other non-specific proteins might be explained by sensitization of the serum donor calf to cell culture proteins at the time of immunization with BRSV.

BRSV subgroup differences were not detected with polyclonal antisera used in this study. Lerch et al. suggested the existence of more than one BRSV subgroup based on a polyclonal Western blot analysis of BRSV strains 391-2 and 127 (10). The G glycoprotein of BRSV strain 391-2 reacted with homologous anti-BRSV strain 391-2 serum, but not with heterologous anti-BRSV strain 127 serum. Unfortunately, BRSV strain 127 was not available for evaluation in our study, so we can neither substantiate nor support this previous report. However, the G glycoprotein of BRSV 391-2 and those of all other BRSV strains tested, reacted with anti-BRSV strain 375 serum, supporting the conclusion that BRSV 391-2 was immunologically related to most other BRSV strains. The BRSV strains that we tested originated from respiratory disease outbreaks in cattle North Carolina, Iowa, Colorado, Texas, and Japan. Possibly, BRSV strain 127, which originated from England, represents a second BRSV subgroup and therefore its G glycoprotein should also differ from the various BRSV strains that we tested. Alternatively, the G glycoprotein specificity of their anti-BRSV strain 127 serum and our anti-BRSV strain 375 serum may have differed. Lerch et al did not test the G glycoprotein specificity of the anti-BRSV strain 127 serum with homologous BRSV strain 127. Inclusion of BRSV strain 127 in our studies might have answered the question of BRSV subgroup differences. Ultimately the molecular characterization of other BRSV G glycoprotein genes will establish whether BRSV subgroup divergence

exists. We have recently cloned and sequenced the G glycoprotein gene from BRSV strain 375 (Duncan, unpublished). The G glycoprotein gene of BRSV strain 127 is currently being characterized (Gail Wertz, personal communication).

Bovine anti-BRSV strain 375 serum in our study did not react with the G glycoprotein of HRSV, CRSV, or ORSV. These data support previous reports suggesting that BRSV represents a third RSV subgroup, distinct from the two HRSV subgroups. CRSV and ORSV may also represent at least one or more additional RSV subgroups, but this was not specifically addressed in this study. Trudel et al found slight serologic cross-reactivity between the G glycoproteins of BRSV and CRSV, but no cross-reactivity between the G glycoprotein of HRSV and BRSV or CRSV (15). In agreement with our work, they concluded that the G glycoprotein of HRSV was different from BRSV and CRSV, but concluded too that the G glycoprotein of BRSV and CRSV were related. We were unable to confirm the antigenic relatedness of G glycoproteins of BRSV and CRSV. This discrepancy might be explained by differences in blotting technique or in antiserum. We loaded identical amounts of total protein contained in infected cell lysates in each lane for polyacrylamide electrophoresis and treated all infected cell lysates similarly, but the proportion of total protein contributed by the G glycoprotein could have varied. Additionally, our BRSV strain 375 antiserum was made in a calf whereas Trudel et al. made their BRSV strain A51908 antiserum in guinea pigs. The CRSV G glycoprotein specificity may have differed in the two sera from two different species. Also, while BRSV strain A51908 may be antigenically related to CRSV, it might not be related serologically to the BRSV strains in our study. Inclusion of BRSV strain A51908 in our study would have addressed this discrepancy.

Since the CRSV, ORSV, and HRSV B8/60 did not react with either antisera, we can not establish a clear distinction among them. However, the work of Orvell et al (14) suggests that there are major differences between the G glycoproteins of CRSV and both HRSV subgroups. To our knowledge, analysis of the structural proteins of ORSV has not been reported. We suspect that ORSV represents an additional RSV subgroup, but we did not specifically address that question in this study. Further studies with anti-HRSV strain B8/60, anti-CRSV, or anti-ORSV sera would test this hypothesis.

The bovine represents a potential model for the study of HRSV because of the clinical, antigenic, and molecular similarities between BRSV and HRSV. Also, a bovine model offers the advantage of studying RSV in its natural host. If more than one BRSV subgroup exists, valuable insight into the importance of subgroup differences in disease pathogenesis and potential immunoprophylaxis could be obtained from a bovine model. Further evaluation of BRSV strains is clearly needed to identify the existence BRSV subgroups. We have started to characterize and compare the G glycoprotein genes among RSV strains. We hope that this work will ultimately lead to better understanding of the role of the G glycoprotein in virion attachment, host range restriction, disease pathogenesis, and immunoprophylaxis.

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PART 3

MOLECULAR CLONING, SEQUENCING, AND COMPARATIVE ANALYSIS OF A BOVINE RESPIRATORY SYNCYTIAL VIRUS (BRSV) G GLYCOPROTEIN GENE

CHAPTER 1

ABSTRACT

Respiratory syncytial virus (RSV) strain and subgroup variation may be important in disease pathogenesis and immunoprophylaxis in cattle. Molecular characterization of the G glycoprotein genes of RSV from different animal species may provide valuable information on subgroup divergence, molecular heterogeneity within RSV subgroups, and host range of RSV strains. Our goal is to compare G glycoprotein genes from several RSV strains by polymerase chain reaction synthesis and base sequencing of their respective cDNAs. In this study, we amplified cDNAs corresponding to the G glycoprotein genes of caprine RSV (CRSV) and bovine RSV (BRSV) strains 391-2 and 375. BRSV strain 375 cDNA was cloned into the Lambda ZAP bacteriophage vector and sequenced by Sanger dideoxy chain termination. The 795 base pair cDNA contained the entire open reading frame of the G glycoprotein gene. This cDNA had considerable nucleotide sequence homology with the published sequence data of BRSV strain 391-2, yet distinct areas of sequence heterogeneity were identified.

CHAPTER 2

INTRODUCTION

Respiratory syncytial virus (RSV) is an important cause of lower respiratory tract disease in both human infants and calves (11) and also has been isolated from respiratory syncytial disease outbreaks in both goats (21) and sheep (12). Respiratory syncytial virus (RSV) is an enveloped virus with a negative sense, single stranded RNA genome (4). The genome has ten genes spread over approximately 15,000 nucleotides; each encodes a unique mRNA (4) transcribed from a single promoter at the 3' end of the genome (5). The mRNAs are translated into ten unique proteins, comprising eight structural and two nonstructural proteins (15). Of the eight structural proteins, two glycoproteins (G and F) constitute the major surface antigens of infected cells and virions (4) and are important immunogenic proteins (7,18,23-25).

Antigenic variation of HRSV has been characterized by immunoreactivity of virus proteins with panels of monoclonal antibodies (1,17), providing a basis for separation of HRSV strains into either subgroup A or B. Variable epitope differences were found in four structural proteins including G, F, M, and N, but the only major differences resided in the G glycoprotein (17). A similar comparison study of HRSV, BRSV, and CRSV strains also showed major antigenic differences between the G glycoproteins of the two human subgroups (19). Additionally, monoclonal antibodies against either human subgroup G glycoprotein did not react with the BRSV or CRSV (19), suggesting that BRSV and CRSV belonged to an additional subgroup(s). Most but not all monoclonal antibodies against the other structural proteins of HRSV subgroup B cross-reacted with HRSV, BRSV, and

CRSV (19). Other investigators have also compared the structural protein profiles of HRSV, BRSV, and CRSV (2,14,22), verifying that distinct antigenic differences exist between the G glycoproteins from HRSV and BRSV (14,22). Serologic cross-reactivity of G glycoproteins from BRSV and CRSV was detected (22). These studies collectively suggest that, in addition to the two human RSV subgroups, BRSV represents an additional distinct RSV subgroup that may be antigenically related to CRSV.

The existence of more than one BRSV subgroup is uncertain because few bovine isolates have been characterized. Lerch, et al reported immunorecognition of BRSV strain 391-2 G glycoprotein by homologous anti-BRSV strain 391-2 serum, but not by heterologous anti-BRSV strain 127 serum, suggesting that like HRSV, BRSV also has antigenic subgroups. We examined, by Western blot analysis, the G glycoproteins of BRSV strains from various geographic sources and compared the immunorecognition of the G glycoproteins from BRSV, HRSV subgroups A and B, CRSV, and ORSV. Bovine anti-BRSV strain 375 serum reacted with the G glycoproteins of all BRSV strains, but not HRSV subgroups A or B, CRSV or ORSV, whereas human anti-HRSV serum reacted with the G glycoprotein of the HRSV strain A2 only. We concluded that, with polyclonal serum immunodetection, we could not identify subgroup differences in the BRSV strains included in our study (Duncan, unpublished).

Although most of the molecular characterization of RSV has come from studies of HRSV, recently BRSV strain 391-2 proteins and mRNAs have been characterized and cDNA clones were derived from viral mRNAs (14). Comparison of the nucleotide sequences of the G glycoprotein genes from BRSV strain 391-2 and HRSV subgroups A and B indicated subgroup divergence of the BRSV G glycoprotein from the two HRSV subgroups (13).

We have begun to further characterize the G glycoproteins of BRSV strains and the two small ruminant strains, CRSV and ORSV. We report here the amplification, cloning, and base sequencing of a cDNA copy of the G glycoprotein gene from BRSV strain 375 and comparisons to other RSV strains.

CHAPTER 3

MATERIALS AND METHODS

Viruses and Cells

Bovine respiratory syncytial virus (BRSV) strain 375 was provided by Dr. Howard Lehmkuhl (National Animal Disease Laboratory, Ames, IA) and bovine turbinate cells (BTU-5) were provided by Dr. Kenny Brock (The Ohio Agricultural Research and Development Center, Wooster, OH). A standard virus preparation was produced by cultivation of BRSV strain 375 on BTU-5 cells with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. Briefly, BTU-5 cells were infected with 0.5 ml of BRSV strain 375 standard preparation when the cell monolayer was 90% confluent. When approximately 50% of the cells in the monolayer had formed syncytia, they were suspended by scraping into the medium and dimethylsulfoxide (DMSO) was added to equal 5% of the total volume as a cryopreservative. One ml aliquots were stored at -70 C. Respiratory syncytial virus infection of BTU-5 cells was confirmed by direct immunofluorescence with a fluoroscein-labelled anti-RSV fusion (F) protein monoclonal antibody (Baxter Healthcare Corp., Bellevue, WA).

RNA Extraction

Bovine turbinate cells (BTU-5) grown in 75 cm^2 tissue culture flasks to 90% confluency, were infected with 0.5 ml of BRSV strain 375 and the virus was allowed to adsorb for 1-3 hours before the medium was replaced with fresh medium. When approximately 50% the cells in the monolayer had formed syncytia, total RNA was

extracted, using a technique modified from that described by Chomczynski and Sacchi (3). Virus-infected cells were scraped into the medium and centrifuged at 1000 g for 10 minutes to pellet the cells. The supernatant was poured off and the pellet was suspended in one ml of solution D (4 M guanidinium thiocyanate; 25 mM sodium citrate, pH 7.0; 0.5% sarcosyl; 0.1 M 2-mercaptoethanol) in a 15 ml polypropylene tube. To this suspension, the following solutions were added sequentially with gentle mixing between each addition: 0.1 ml 2 M sodium acetate, pH 4.0; 1.0 ml water-saturated phenol; 0.2 ml chloroform-isoamyl alcohol, 49:1. The mixture then was shaken vigorously for ten seconds and then placed on ice for fifteen minutes. The sample was divided into two equal volumes and placed in 1.7 ml microcentrifuge tubes and spun in a centrifuge at 10,000 g and 4 C for 20 minutes. The aqueous phase was transferred to a clean tube, one ml of isopropanol was added to it and held at -20 C for at least one hour to precipitate the RNA. The tube was the spun in a centrifuge at 10,000 g and 4 C for 20 minutes. The RNA pellet was dissolved in 0.3 ml of solution D, 1 ml of isopropanol was added and held at -20 C for at least one hour. The RNA was pelleted again by centrifugation at 10,000 g and 4 C for 20 minutes. The supernatant was removed and the pellet was washed with 70% ethanol. The pellet was dried in a vacuum concentrator (Savant Instruments, Inc., Farmingdale, NY) for approximately 5 minutes. The RNA was resuspended in 50-100 μl of diethyl pyrocarbonate-treated distilled water and the RNA was quantified by measuring absorbance at 260 nm in a spectrophotometer (Lambda 3B, UV/VIS, Perkin-Elmer Cetus, Norwalk, CT).

Oligonucleotide Primer Design

Design of oligonucleotide primers for polymerase chain reaction amplification of the BRSV strain 375 G glycoprotein gene was based on the published sequence of the G glycoprotein gene of BRSV strain 391-2 (Lerch et al). These primers were designed to amplify cDNA product from BRSV strain 375 genomic RNA and include restriction endonuclease sites so that the resultant cDNA could be unidirectionally inserted into the Lambda ZAP bacteriophage cloning vector (Stratagene, La Jolla, CA). The BRSV strain 391-2 G glycoprotein gene sequence reported by Lerch et al (13) was analyzed with Micro Genie Sequence Analysis Program computer software (Spinco Division, Beckman Instruments, Inc., Palo Alto, CA) to rule out the presence of any EcoR 1 or Xho 1 restriction endonuclease sites within the sequence that would confound cloning attempts. Selection of primers was based on standard principles of efficient primer design; 18-28 nucleotides in length, 50-60% G+C content, avoidance of sequences with secondary structure, avoidance of complementarity at the 3' ends of primers (8). Oligonucleotide primers BD-1 and BD-2 were synthesized by Oligos Etc. Inc., Guilford, CT. Primer 1 (BD-1) contained 30 nucleotides (5' CCGAATTCCAAGTATGTCCAACCATACCCA 3') and was designed to hybridize to the 3' end of the negative sense RNA G glycoprotein gene and also provide the EcoR 1 restriction endonuclease site. Primer 2 (BD-2) contained 28 nucleotides (5' GGCTCGAGCTAGATCTGTGTAGTTGATT 3') and was designed to hybridize to the 3' end of the positive sense strand cDNA from the cDNA first strand synthesis and provide the Xho 1 restriction endonuclease site. Because some restriction endonucleases fail to cleave at sequences located near the

extreme ends of DNA fragments (10), two cytidine 5' triphosphate and two guanidine 5' triphosphate nucleotide residues were incorporated at the 5' ends of BD-1 and BD-2 oligonucleotide primers respectively, immediately upstream from the restriction endonuclease sites. Figure 1 depicts primers BD-1 annealing to the complimentary negative-sense single stranded RNA genome of BRSV strain 391-2. Primer BD-2 is also depicted; it is homologous to the 5' end of the G glycoprotein gene and it is designed to anneal to the cDNA following first strand synthesis. With this strategy, we could insure directional insertion of the amplified cDNA into the multiple cloning site of the Lambda ZAP vector.

Polymerase Chain Reaction Amplification

The G glycoprotein gene from BRSV strain 375 was amplified by the polymerase chain reaction according to a protocol described by Perkin-Elmer Cetus Corp., Norwalk, CT. We optimized the amplification procedure, by holding total RNA constant and selecting 3 primer and 5 $MgCl_2$ concentrations.

The initial amplification step was to synthesize a single strand cDNA copy of the G glycoprotein gene using Maloney Murine Leukemia Virus (MMLV) reverse transcriptase according to the manufacturer's instructions (Gibco BRL, Grand Island, NY). Briefly, 2 μg of total RNA from BRSV 391-2, BRSV 375, CRSV, ORSV, or bovine viral diarrhea virus (BVDV) strain 72 infected cells or total RNA from uninfected BTU-5 cells was placed in a microcentrifuge tube and the following were added sequentially and mixed: 200 μM of each 2'-deoxynucleoside 5'-triphosphate (dATP, dCTP, dGTP, and dTTP) (Gibco BRL, Grand Island, NY); 20 units of RNasin (Promega, Madison, WI); 10 mM DTT;

Figure 1. Oligonucleotide primer design. This schematic representation of the design strategy of primers BD-1 and BD-2. BD-1 is designed to anneal to the single stranded RSV genomic RNA and prime first strand cDNA synthesis. The oligonucleotide, GAATTC, within the parallelogram indicates the pallindromic sequence of the EcoR 1 restriction endonuclease site. BD-2 is designed to anneal to the positive sense cDNA resulting from first strand synthesis and prime the complimentary negative sense second cDNA strand. The oligonucleotide, GAGCTC, within the parallelogram indicates the pallindromic sequence of the Xho 1 restriction endonuclease site. Nucleotide triplets that correspond to the start and stop codons of the mRNA are indicated by labelled boxes.

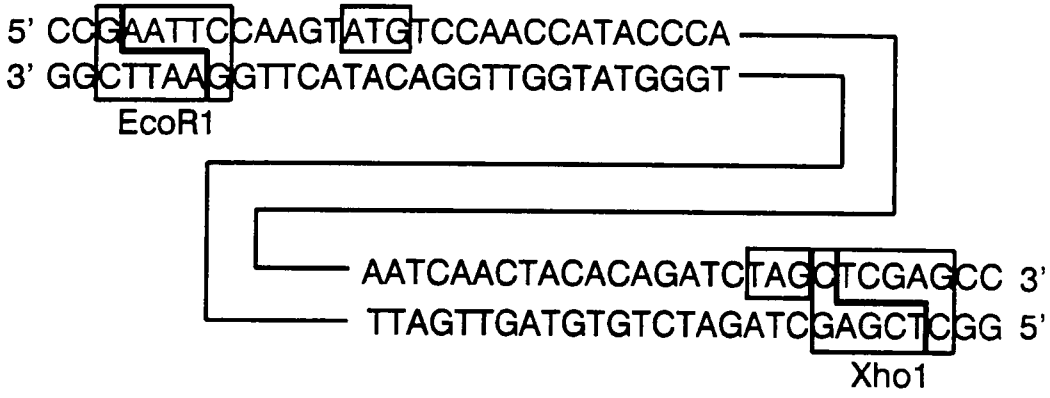

5' CC GAATTC CAAGTATGTCCAACCATACCCA 3'
BD-1 primer (+)
3' GTTCATACAGGTTGGTATGGGT
start
BRSV 391-2 negative sense RNA genome
TTAGTTGATGTGTCTAGATC 5'
stop
TTAGTTGATGTGTCTAGATC GAGCTC GG
BD-2 primer (-)

primer 1 (BD-1) (0.2, 1.0, or 2.0 μM); 400 units of MMLV reverse transcriptase (Gibco BRL, Grand Island, NY); 10 μl of reverse transcriptase buffer concentrate (Gibco BRL, Grand Island, NY); and deionized water to make a final volume of 50 μl. The mixture was incubated at 37 C for one hour and then placed on ice.

Thermostable Taq (Thermus aquaticus) DNA polymerase was used to amplify the single strand cDNA according to the manufacturer's instructions (Perkin-Elmer Cetus Corp., Norwalk, CT). The following were added to the reverse transcriptase mixture sequentially and mixed: 100 μM of each 2'-deoxynucleoside 5'-triphosphates (dNTPs)(Gibco BRL, Grand Island, NY); primer 2 (BD-2) (0.1, 0.5, or 1.0 μM); 10 μl of PCR buffer 10X (100 mM Tris-HCl, pH 8.3; 500 mM KCl; 10, 20, 30, 35, or 40 mM $MgCl_2$; 0.1% gelatin); 2.5 U of Taq polymerase; and deionized water to make a final volume of 100 μl.

The reaction mixture was held at various tempertures in a model GTC-1 automatic thermal cycler (Precision Scientific, Inc., Chicago, IL) using the following conditions: 50 C for 2 minutes (annealing reaction); 72 C for 3 minutes (extension reaction); and 94 C for 1 minute (denaturation). This temperature cycle was repeated 25 times ending with a final annealing and extension period of 2 minutes and 10 minutes respectively. Figure 2 depicts amplified target double stranded cDNA following PCR thermocycling. Ten μl of the PCR reaction mixture was mixed with 2 μl of tracking dye (0.25% bromphenol blue; 0.25% xylene cyanol FF; 15% Ficoll (type 400; Pharmacia) (in water) and resolved by 1% horizontal agarose gel electrophoresis with TBE buffer (0.09 M Tris base; 0.09 M boric acid; 0.002 M EDTA) and 0.5 μg/ml ethidium bromide in a model MPH horizontal electrophoresis unit (International Biotechnologies, Inc., New Haven, CT). Size estimates of products were determined by comparing their electrophoretic

Figure 2. Polymerase chain reaction G glycoprotein gene amplified target cDNA. Depicted, is a schematic representation of amplified target cDNA following PCR using primers BD-1 and BD-2 and genomic RNA from respiratory syncytial virus. Restriction endonuclease sites EcoR 1 and Xho 1 are labelled and nucleotide triplets corresponding to the start and stop codons of the G glycoprotein gene mRNA are indicated by boxes.


5' CCGAATTCCAAGTATGTCCAACCATACCCA
3' GGCTTAAGGTTCATACAGGTTGGTATGGGT
EcoR1
AATCAACTACACAGATCTAGCTCGAGCC 3'
TTAGTTGATGTGTCTAGATCGAGCTCGG 5'
Xho1

migration with molecular weight standards (123 base pair ladder, Gibco BRL, Grand Island, NY).

Purification of cDNA Fragment

Approximately 60 μl of PCR reaction mixture with 2 μl of tracking dye was loaded into two adjacent lanes (30 μl each) of a 1% horizontal agarose gel with 0.5 μg/ml ethidium bromide and TBE buffer. The cDNA was isolated by electrophoresis at 100 volts constant voltage. When the bromphenol blue had run half the length of the gel, the gel was inspected with superficial ultraviolet (UV) light and the appropriate band was identified. A scalpel incision was made just to the anode side of the band of interest. A piece of NA45 paper (Schleicher and Schuell, Inc., Keene, NH), premoistened in TBE buffer, was inserted in the incision and electrophoresis was continued for 10-15 minutes until the cDNA band completely stacked onto the NA45 paper. The paper was then moved to a 1.7 ml ultracentrifuge tube containing 400 μl of elution buffer (1 M sodium chloride; 0.5 M arginine free base, pH 9.0) and incubated for one hour at 65 C to elute cDNA from the paper. The cDNA was extracted from the eluate with addition of an equal volume of phenol/chloroform and following centrifugation at 10,000 g for ten minutes, the aqueous phase was transferred to a separate tube and two volumes of 100% ethanol were added. The tube was held at -20 C overnight and centrifuged at 10,000 g the following day. The cDNA pellet was washed in 70% ethanol and dried in a vacuum concentrator for approximately five minutes.

Restriction Endonuclease Digestion of Amplified cDNA

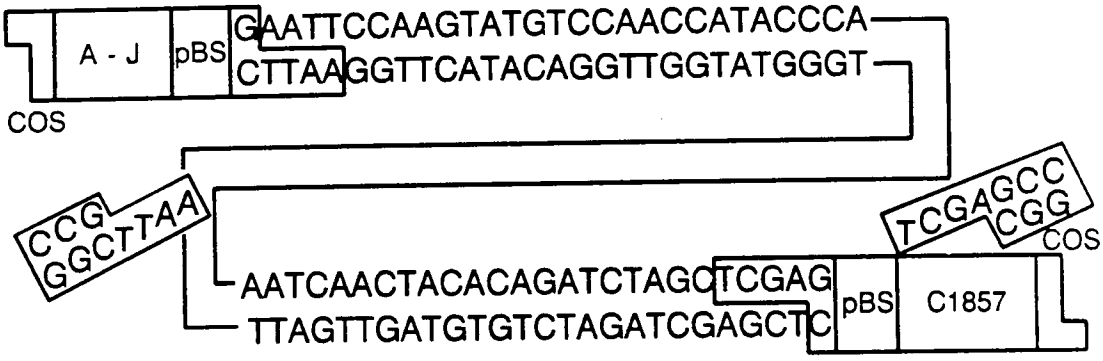
The dried cDNA pellet was resuspended in 35 μl of distilled water and the following were added and mixed sequentially: 5.0 μl concentrated restriction endonuclease buffer C (International Biotechnologies, Inc., New Haven, CT), 2.5 μl EcoR 1 (16 U/μl) (International Biotechnologies, Inc., New Haven, CT), 2.5 μl Xho 1 (12 U/μl) (International Biotechnologies, Inc., New Haven, CT), and 5.0 μl of acetylated bovine serum albumin (BSA)(Promega, Madison, WI). The digestion mixture was incubated at 37 C for one hour. Following enzyme digestion, the cDNA was extracted by adding an equal volume of phenol/chloroform. After centrifugation at 10,000 g for ten minutes, the cDNA in the aqueous phase was transferred to a clean tube and 0.1 volume of 3 M sodium acetate was added and the cDNA was precipitated overnight at -20 C after adding two volumes of 100% ethanol. The precitated cDNA was pelleted by centrifugation at 10,000 g for ten minutes, the supernatant removed, and the pellet was washed with 70% ethanol before drying in a vacuum concentrator for five minutes.

Molecular Cloning of Amplified cDNA Fragment into Lambda ZAP Vector

Restriction endonuclease-digested cDNA was resuspended in 15 μl of distilled water and ligated into the pre-digested Uni-ZAP XR vector arms according to manufacturer's instructions (Stratagene, La Jolla, CA). In a 0.6 ml microfuge tube, the following were added in sequence: 2.5 μl of resuspended cDNA; 0.5 μl concentrated ligation buffer; 0.5 μl 10 mM rATP; 1.0 μl Uni-ZAP XR vector arms (1 μg/μl); and 0.5 μl T4 DNA ligase (4 Weiss U/μl). The ligation mixture was

incubated for two days at 4 C and then at room temperature for two hours. Figure 3 depicts the ligation of PCR-generated amplified target cDNA into the multiple cloning site of lambda ZAP bacteriophage DNA. Twenty-four hours before the lambda bacteriophage library was to be packaged, a 50 ml culture of PLK-F' bacterial cells in LB broth (NaCl, 10 g/l; Bacto-Tryptone, 10 g/l; Yeast extract, 5 g/l; supplemented with 0.2% maltose and 10 mM supplemented with 0.2% maltose and 10 mM magnesium sulfate) of PLK-F' cells was started from a colony isolated on a LB/tetracycline (12.5 μg/ml) agar. Approximately 50,000 recombinant bacteriophage were mixed with 600 μl of PLK-F' cells ($O.D._{600}$ = 0.5) and incubated at 37 C for 15 minutes before adding 6.5 ml of 48 C top agar and plating on 150 mm NZY agar plates that had been prewarmed to 37 C. The agar plates then were incubated for 5-8 hours at 37 C. Ten ml of SM buffer were added to each plate and they were incubated on an oscillating platform at 4 C overnight. On the next day, the bacteriophage suspension was recovered from the plates and the plates were washed with an additional 2 ml of SM buffer and pooled with the suspension. Chloroform (to equal 5% of the suspension volume) was added to the pooled suspension and cell debris was removed by centrifugation for five minutes at 4000 g. The supernatant fluid was transferred to sterile polypropylene tubes and chloroform was added to equal 0.3% of the total volume. The amplified bacteriophage library was quantified as described above assuming approximately 1010-1012 pfu/ml. The recombinant bacteriophage library was stored at 4 C.

Figure 3. Ligation of amplified cDNA into Lambda ZAP bacteriophage vector. Depicted, is a schematic representation of ligation of amplified G glycoprotein gene cDNA into the left (COS, A-J) and right (C1857, COS) arms of the lambda ZAP bacteriophage vector DNA. The angled boxes depict the cleavage fragments following restriction endonuclease EcoR 1 and Xho 1 digestion of PCR amplified G glycoprotein gene target cDNA. Boxes labelled pBS indicate sequences of the pBluescript phagemid used in *in vivo* excision.


A - J
pBS
GAATTCCAAGTATGTCCAACCATACCCA
CTTAAGGTTCATACAGGTTGGTATGGGT
COS
CCG
GGCTTAA
TCGAGCC
CGG
COS
AATCAACTACACAGATCTAGCTCGAG
TTAGTTGATGTGTCTAGATCGAGCTC
pBS
C1857

Screening of Recombinant Lambda ZAP Bacteriophage Library

The screening of the bacteriophage library involved two steps; a blue/white color selection of bacteriophage plaques was followed by polymerase chain reaction. Twenty-four hours before the library was to be screened, a 50 ml culture of XL1-Blue cells in LB broth (NaCl, 10 g/l; Bacto-Tryptone (Difco Laboratories, Detroit, MI), 10 g/l; Yeast extract, 5 g/l supplemented with 0.2% maltose and 10 mM magnesium sulfate) was started and incubated at 37 C. Approximately 22,000 recombinant bacteriophage were mixed with 200 μl of XL1-Blue cells ($O.D._{600}$ = 0.5) and incubated at 37 C for 15 minutes before adding 2-3 ml of 48 C top agar and plating on 100 mm NZY plates that had been prewarmed to 37 C. The top agar also contained IPTG (isopropylthio-β-D-galactoside) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside): 40 μl of a X-gal concentration solution (X-gal, 20 mg/ml in dimethylformamide) and 8 μl of a IPTG concentrated solution (IPTG, 100 mg/ml in distilled water) were added to the warm top agar for each 100 mm plate. Plates were incubated overnight at 37 C and recombinant white plaques produced by recombinant phage were identified.

Each white plaque was transferred to 0.6 ml microcentrifuge tube containing 500 μl of SM buffer and 20 μl chloroform, mixed vigorously and incubated at room temperature for 1-2 hours to elute the phage. This phage preparation was quantified as described previously and stored at 4 C.

Five μl of each phage eluate, 0.5 M of each primer (BD-1 and BD-2), and 2.5 mM $MgCl_2$ were used for polymerase chain reaction screening according to the procedure described previously.

In Vivo Excision of Recombinant pBluescript Plasmid

Twenty-four hours before the pBluescript plasmid was to be excised from the recombinant phage, a 50 ml culture of XL1-Blue cells in LB broth was incubated overnight at 37 C. In a 50 ml conical tube the following were mixed: 200 μl of XL1-Blue cells ($O.D._{600}$ = 1.0) in 10 mM $MgSO_4$, 200 μl of Uni-ZAP XR phage preparation containing approximately 10,000 phage particles, and 1 μl of R408 helper phage (> $1x10^6$ pfu/ml, Stratagene, La Jolla, CA) and incubated at 37 C for 15 minutes. Five ml of concentrated LB (NaCl, 20 g/l; Bacto-Tryptone, 20 g/l; Yeast extract, 10 g/l) medium was added to each tube and tubes were incubated for an additional 3 hours at 37 C with gentle shaking. The tubes then were heated to 70 C for 20 minutes and spun in a centrifuge for five minutes at 4000 g. Supernatant fluid, containing encapsidated pBluescript phagemid, was decanted to separate sterile tubes and stored at 4 C for up to 1-2 months. Two hundred μl of encapsidated phagemid preparation and 20 μl of a 1:100 dilution of phage stock were each mixed with 200 μl XL1-Blue cells ($O.D._{600}$ = 1) and incubated at 37 C for 15 minutes. One, 5, 10, 20, 50, and 100 μl of encapsidated phagemid/bacteria mixture and diluted encapsidated phagemid/bacteria mixture were spread on LB/ampicillin plates and incubated overnight at 37 C. Colonies appearing on the plates consisted of bacteria containing double stranded phagemid, because the phagemid confered ampicillin resistance. Glycerol storage preparations of bacteria containing recombinant phagemid were prepared by inoculation of super broth (NaCl, 5g/l; Bacto-yeast extract, 20 g/l; Tryptone, 35 g/l; and 5 M NaOH, 1 ml/l) with bacteria containing phagemid, incubation at 37 C in an oscillating shaker overnight, addition of sterile glycerol to equal 15% of the total volume, and stored at -70 C.

Preparation of Plasmid DNA for Sequencing

Small amounts of plasmid DNA for sequencing were prepared with a modification of the method described by Morelle (16). Two ml of super broth, in a sterile 15 ml polypropylene tube, was inoculated with XL1-Blue bacteria containing phagemid, selected from a colony grown on LB/ampicillin agar and incubated overnight at 37 C. The broth culture was transferred to a 2.0 ml microfuge tube, spun in a centrifuge at 10,000 g for ten minutes, and the supernatant fluid was poured off and the pellet saved. The cell pellet was resuspended in 200 μl of TSE lysis buffer (50 mM glucose; 25 mM Tris-HCl, pH 8.0; 10 mM EDTA) and lysozyme was added to a final concentration of 4 mg/ml and the mixture was incubated for five minutes at room temperature. Then, 400 μl of alkaline sodium dodecyl sulfate (SDS) solution (0.2 N NaOH, 1% sodium dodecyl sulfate) was added to the above mixture, mixed gently, and the mixture was held on ice for five minutes. Then, 300 μl of 7.5 M ammonium acetate was added to the above mixture, mixed gently, and incubated on ice for ten minutes. The tube was then spun in a centrifuge at 10,000 g for 15 minutes and the supernatant was carefully transferred to a separate tube and isopropanol was added to equal 60% of the total volume and the tube was incubated at room temperature for ten minutes. Following centrifugation at 10,000 g for 30 minutes, the supernatant was removed and the pellet was washed with 70% ethanol and dried in a vacuum concentrator for five minutes.

Alkaline Denaturation of Plasmid DNA

Denaturation of plasmid DNA was done using a procedure described by Promega (Promega, Madison, WI). The dried plasmid DNA pellet from above (see Preparation of Plasmid DNA for Sequencing) was resuspended in 18 μl of deionized water. Two μl of a 2 M NaOH, 2 mM EDTA solution was added and the tube was incubated at 37 C for 30 minutes. Two μl of 2 M ammonium acetate, pH 4.6 was added and the tube was mixed on a vortex mixer. Seventy-five μl of 100% ethanol was added and the contents of the tube were mixed on a vortex mixer. The tube then was placed at -70 C for ten minutes followed by centrifugation at 10,000 g for ten minutes. The denatured DNA pellet was washed with 70% ethanol and dried in a vacuum concentrator for five minutes.

Dideoxy Chain Termination Sequencing

Sequencing of the recombinant pBluescript plasmid DNA was done according to manufacturer's instructions (Sequenase version 2.0, United States Biochemical, Corp., Cleveland, OH).

The following buffers were provided in the Sequenase kit and stored at -20 C.

1. Sequenase buffer concentrate (200 mM Tris-HCl, pH 7.5; 100 mM $MgCl_2$; 250 mM NaCl);
2. Dithiothreitol, 0.1 M;
3. Labeling Mix concentrate (7.5 μM dGTP; 7.5 μM dCTP; 7.5 uM dTTP);

4. ddG Termination Mix (80 μM dGTP; 80 μM dATP; 80 μM dCTP; 80 μM dTTP; 8 μM ddGTP; 50 mM NaCl);

5. ddA Termination Mix (80 μM dGTP; 80 μM dATP; 80 μM dCTP; 80 μM dTTP; 8 μM ddATP; 50 mM NaCl);

6. ddT Termination Mix (80 μM dGTP; 80 μM dATP; 80 μM dCTP; 80 μM dTTP; 8 μM ddTTP; 50 mM NaCl);

7. ddC Termination Mix (80 μM dGTP; 80 μM dATP; 80 μM dCTP; 80 μM dTTP; 8 μM ddCTP; 50 mM NaCl);

8. Sequence Extending Mix (180 μM each of dATP; dGTP; dCTP; dTTP; 50 mM NaCl);

9. Sequenase Version 2.0 Enzyme;

10. Enzyme Dilution Buffer (10 mM Tris-HCl, pH 7.5; 5 mM dithiothreitol (DTT); 0.5 mg/ml bovine serum albumin (BSA);

11. Stop Solution (95% Formamide; 20 mM EDTA; 0.05% bromphenol blue; 0.05% xylene cyanol FF)

Additionally, [α-^{35}S]-dATP 0.250 mCi (Dupont NEN Products, Boston MA) was used for autoradiographic detection of primer extension sequencing products.

Four primers were used for sequencing the entire G glycoprotein gene. T3 and T7 primers (Promega, Madison, WI) were used for sequencing the positive and negative strands, respectively, of the 5' and 3' ends of the cDNA insert. Two other oligonucleotide primers (5' TTACCTTCACATGTACTGCA 3' and 5' TAACCTTGACATCACTCGTC 3', synthesized by Biosynthesis, Inc., Denton, TX), derived from sequence data obtained using the T3 and T7 primers, were used to sequence positive and negative strands, respectively, of internal regions of the G glycoprotein gene cDNA.

The DNA from the alkaline denaturation step was resuspended in a 0.6 μl microcentrifuge tube in 7 μl of deionized water and 2 μl of Sequenase buffer concentrate and 1 μl of the appropriate primer was added and mixed by pipetting. The tube was heated to 65 C for two minutes and then cooled slowly (over a period greater than fifteen minutes) to less than 35 C to anneal the primer onto the template, before placing on ice. While the tube was cooling, 2.5 μl of each termination mixture was placed, respectively, in one of four separate microcentrifuge tubes, labelled G, A, T, and C. Also, at the same time the annealing mixture was cooling, 2 μl of Labeling Mixture was transferred into a separate microcentrifuge tube (labelled L) and diluted 1:5 with 8 μl of deionized water. In another microcentrifuge tube (labelled E), 7 μl of Enzyme Dilution Buffer and 1 μl of Sequenase Version 2.0 were mixed. The four tubes containing the four different termination mixtures were warmed to 37 C. When the annealing mixture had cooled to less than 35 C, 1 μl of DTT, 2 μl of diluted labelling mixture and 0.5 μl of [α-^{35}S]-dATP, and 2 μl of diluted Sequenase version 2.0 were added sequentially, and the labelling reaction mixture was incubated at room temperature for 2-5 minutes. Then, 3.5 μl of labelling reaction mixture was added to each of the four termination mixture tubes and the termination reactions were incubated at 37 C for five minutes. Finally, each of the four termination reaction tubes were stopped with the addition of 4 μl of Stop Solution. Each reaction mixture was stored at -20 C until loaded onto a 6% polyacrylamide/7M urea gel for electrophoretic separation. Each reaction mixture was heated to 75 C for two minutes prior to loading onto the gel.

Electrophoresis of sequencing reaction mixtures was done using the following solutions:

1. Tris-borate-EDTA (TBE) buffer (10X concentrate) (109 g/l Tris base; 55 g/l boric acid; 9.6 g/l EDTA)

2. Modified TBE buffer (20X concentrate) (157.4 g/l Tris base; 27.8 g/l boric acid; 9.6 g/l EDTA)

3. Polyacrylamide/Urea Stock Solution (urea, 233.5 g/500 ml; acrylamide, 28.5 g/500 ml; bis-acrylamide 1.5 g/500 ml; 10X TBE, 50 ml/500 ml)

4. DNA Sequencing Gel (Polyacrylamide/Urea Stock Solution, 100 ml; 10% ammonium persulfate in water, 670 μl; N, N, N', N', tetramethyl-ethylenediamine (TEMED) (20 μl)

5. DNA Sequencing Gel Fixing Solution (5% methanol, 5% acetic acid)

Two and one half μl of each of four sequencing reaction tubes were loaded onto a polyacrylamide/urea gel and separated at 2000 volts (constant voltage) on a vertical electrophoresis unit (Base Runner Nucleic Acid Sequencing Unit, International Biotechnologies, Inc., New Haven, CT) according to the manufacturer's instructions. Generally, the four reaction mixtures were loaded onto the gel twice, three hours apart in four consecutive lanes labelled A, C, G, and T. A total running time of six hours was necessary to obtain maximum sequence information. Following the completion of electrophoretic separation, the gel was transferred to blotting paper and fixed in 1 liter of fixing solution for 20 minutes. After thorough draining, the gel was placed in a gel dryer (Bio-Rad model 583, Bio-Rad Laboratories, Richmond, CA) according to manufacturer's instructions for 90 minutes. Dried gels were placed in cassettes with 35 x 43 cm Kodak X-OMAT AR

film (Eastman Kodak Co., Rochester, NY) for autoradiographic exposure for 48 hours before developing in a Kodak X-OMAT model 460 RA automatic film processor (Eastman Kodak Co., Rochester, NY). Sequence data was read by hand with the aid of a light box (model 460 A, S & S X-ray Products, Brooklyn, NY).

Analysis of Sequence Data

Sequence data were analysed and compared to other published sequences with Microgenie Sequence Analysis Program computer software (Spinco Division, Beckman Instruments, Inc., Palo Alto, CA).

CHAPTER 4

RESULTS

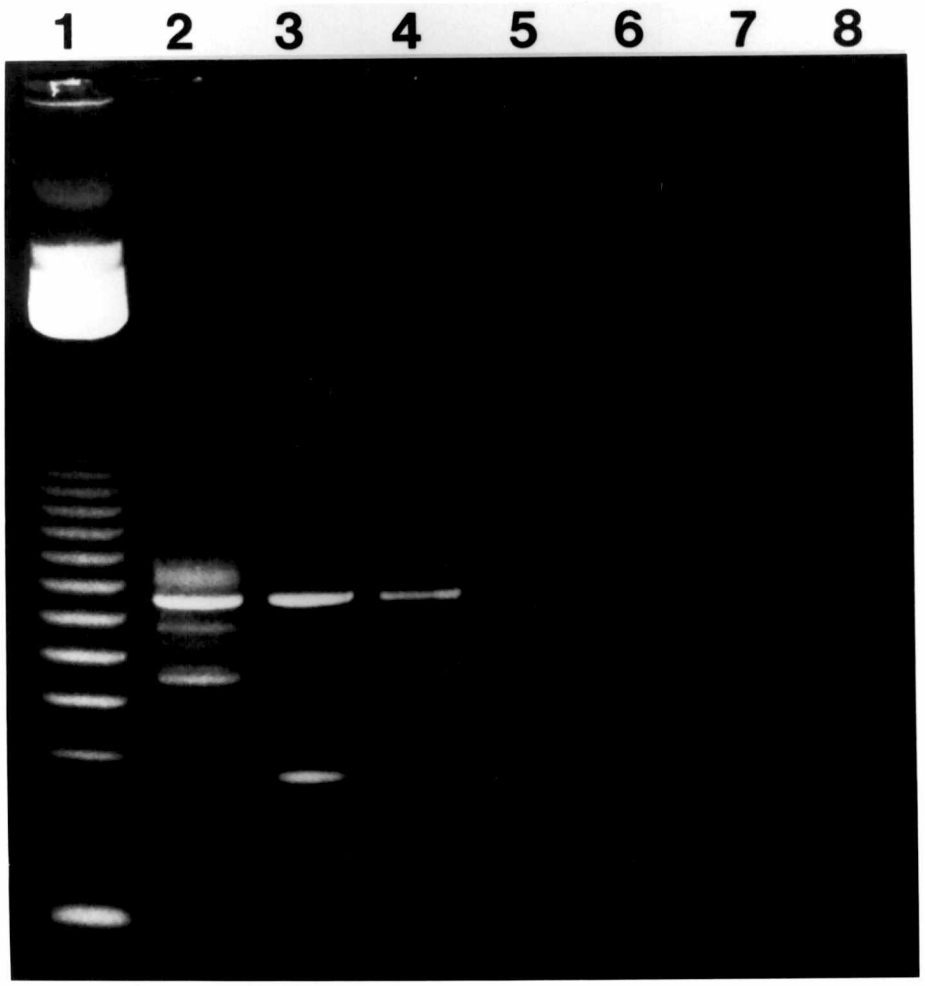
Polymerase Chain Reaction Analysis

Using primers BD-1 and BD-2, we amplified several cDNA species of various sizes from BRSV strains 391-2 and 375 and CRSV, but not ORSV (Figure 4). Based on the published G glycoprotein gene sequence from BRSV strain 391-2, we expected to amplify a target cDNA fragment of 795 base pairs in length. cDNA products of the appropriate size were produced using RNA from cells infected with BRSV strain 391-2, BRSV strain 375, and CRSV. The PCR products using RNA from BRSV strain 391-2 infected cells included the 795 base pair target that we expected plus additional bands of the following approximate sizes: 183, 328, 553, 574, 656, 697, and 880 base pairs. The PCR products using BRSV strain 375 RNA included the 795 base pair target plus 328 and 880 base pair products. The PCR products using CRSV RNA included the a 795 base pair target plus 328, 553, 697, and 880 base pair products. There were no PCR amplified cDNA products evident following PCR amplification using RNA from ORSV infected cells. Polymerase chain reaction analyses using RNA from bovine viral diarrhea virus (BVDV) strain 72 infected cells, RNA from uninfected BTU-5 cells, or no RNA served as negative controls and amplification of target cDNA did not occur.

Production of Recombinant Bacteriophage

Polymerase chain reaction-generated BRSV strain 375 G glycoprotein gene cDNA was cloned into the Lambda ZAP bacteriophage vector. Recombinant

Figure 4. Polymerase chain reaction analysis of various respiratory syncytial virus strains. This is an ethidium bromide-stained agarose gel of polymerase chain reaction analysis products using primers BD-1 and BD-2 and RNA from BTU-5 cells infected with: BRSV strain 391-2, (lane 2); BRSV strain 375, (lane 3a); CRSV, (lane 4); ORSV, (lane 5); and BVDV strain 72, (lane 7). Lanes 6 and 8 contained polymerase chain reaction analysis products using no RNA target (lane 6) and RNA from uninfected BTU-5 cells (lane 8). Lane 1 contains molecular size standard markers (123 base pair DNA ladder, Gibco BRL, Gaithersburg, MD).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bacteriophage plaques, identified by blue/white color selection, were screened by PCR with primers BD-1 and BD-2, to insure that the recombinant bacteriophage contained DNA inserts of the appropriate size (795 base pairs). Figure 5 illustrates PCR-generated cDNA amplification products from recombinant bacteriophage plaques. The 795 base pair cDNA band was consistent with the expected cDNA target insert length. Following *in vivo* excision of pBluescript phagemid from lambda bacteriophage infected bacterial cells, the pBluescript phagemid was screened by digestion of purified phagemid with restriction endonucleases EcoR 1 and Xho 1 to verify the presence of a 795 base pair insert (Figure 6).

Sequence Analysis

Sequencing of our cDNA in the recombinant phagemid confirmed its 795 base pair length inclusive of the entire coding region (774 bases), 5 bases upstream of the start codon at the 5' end, and 12 bases contributing to the enzyme restriction sites at the 5' and 3' ends (Figure 7). The coding region of our G glycoprotein gene cDNA was identical in length to the published sequence of the coding region of the G glycoprotein gene of BRSV strain 391-2 (13). In contrast, the coding region of our cDNA differed in length from the coding regions of HRSV strains A2 and 18537, which were 897 and 879 bases in length respectively (13). Because our primers were designed to amplify the coding region of the G glycoprotein gene only, we could not analyze the noncoding regions at either end of the gene. The coding region of our cDNA shared 95.0% nucleotide sequence identity with the coding region of BRSV strain 391-2, and 48.3% and 49.2% nucleotide sequence identity with the coding regions from published sequences of HRSV strains A2 and 18537 respectively.

Figure 5. Polymerase chain reaction analysis of recombinant lambda bacteriophage. This is an ethidium bromide-stained agarose gel of polymerase chain reaction analysis products of recombinant lambda bacteriophage plaques. A single cDNA product with an approximate molecular size of 795 base pairs in lanes 2, 3, and 4 confirms the presence of specific target cDNA in recombinant lambda bacteriophage. No PCR products were generated from other recombinant lambda bacteriophage (lanes 5-8). Lane 1 contains molecular size standard markers (123 base pair DNA ladder, Gibco BRL, Gaithersburg, MD).

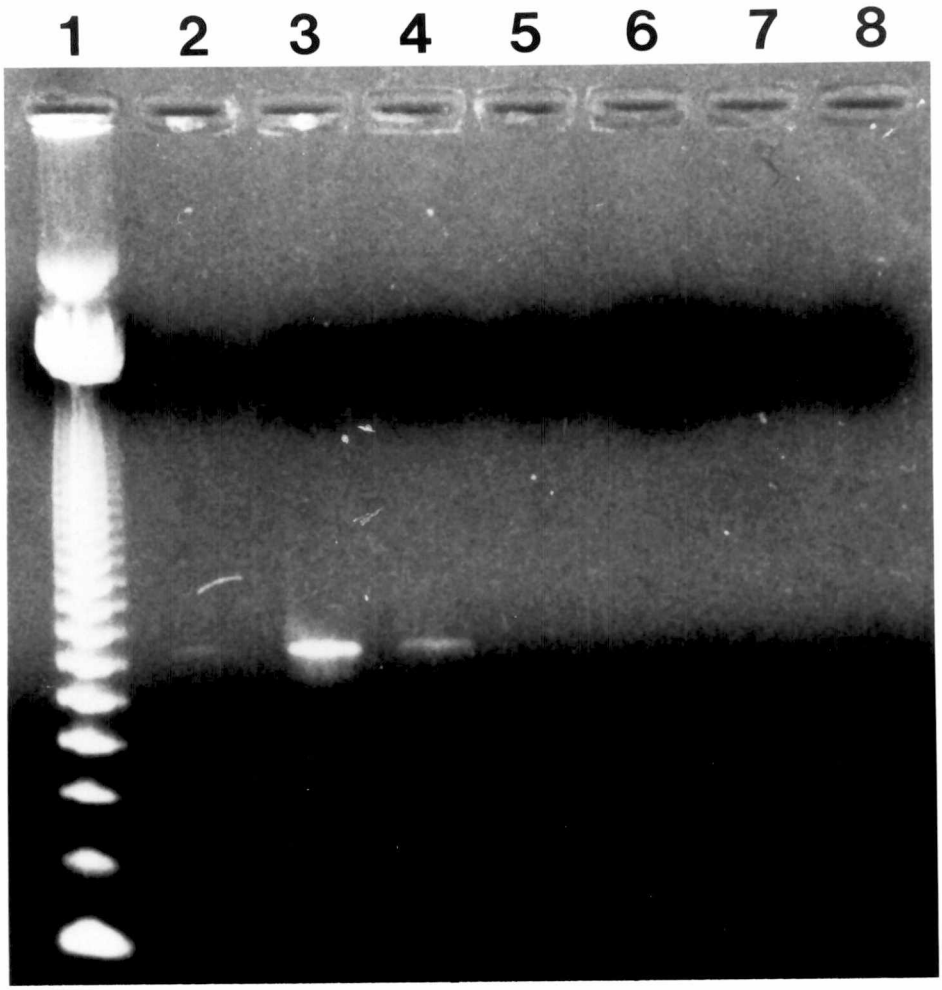
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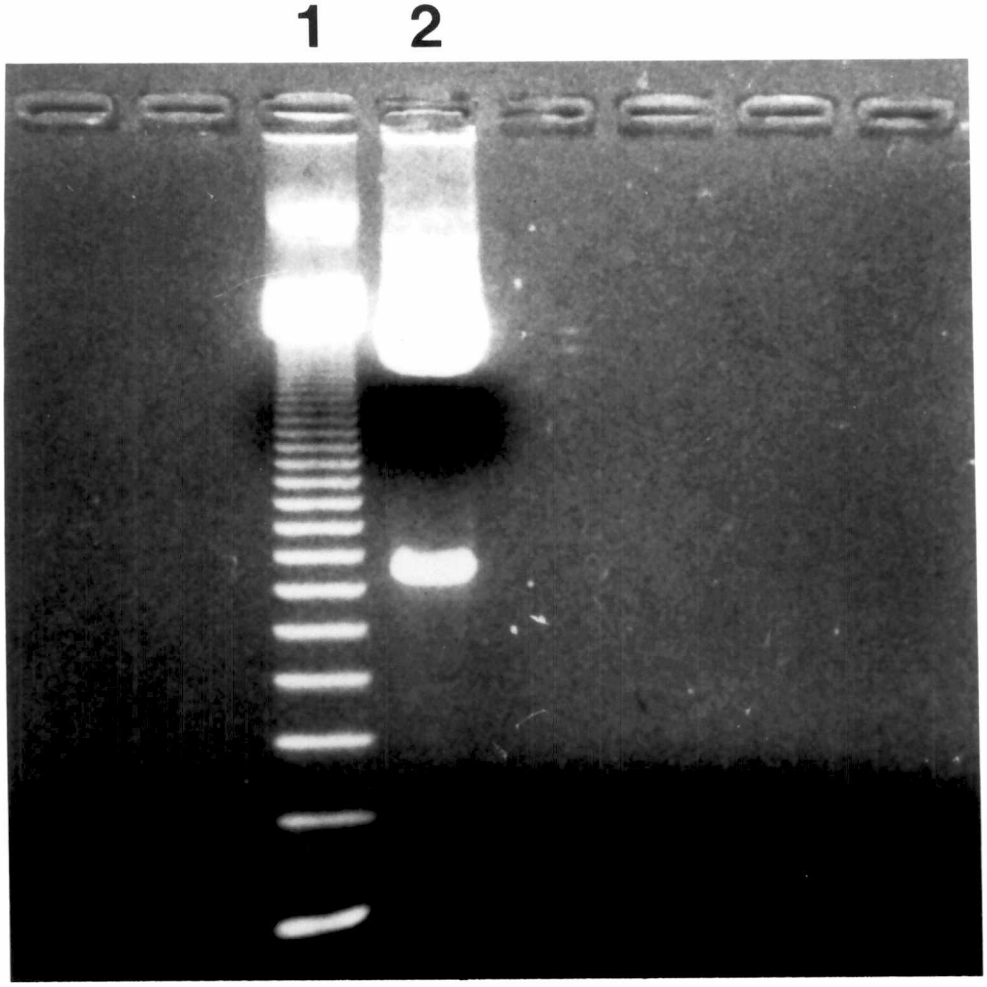
Figure 6. Restriction endonuclease EcoR 1 and Xho 1 digestion products of recombinant pBluescript phagemid. This ethidium bromide-stained agarose gel has a single cDNA species of approximately 795 base pairs length (lane 2) confirming the presence of specific target cDNA in recombinant phagemid. Lane 1 contains molecular size standard markers (123 base pair DNA ladder, Gibco BRL, Gaithersburg, MD).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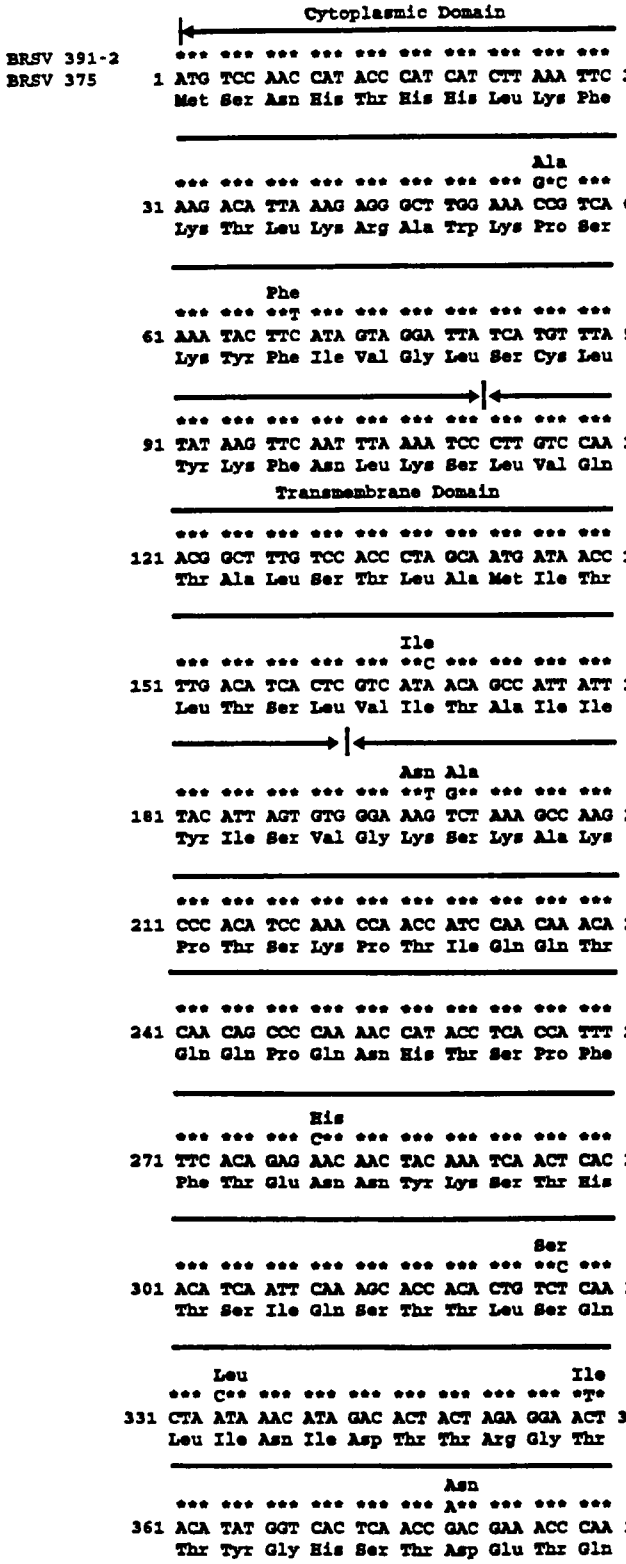
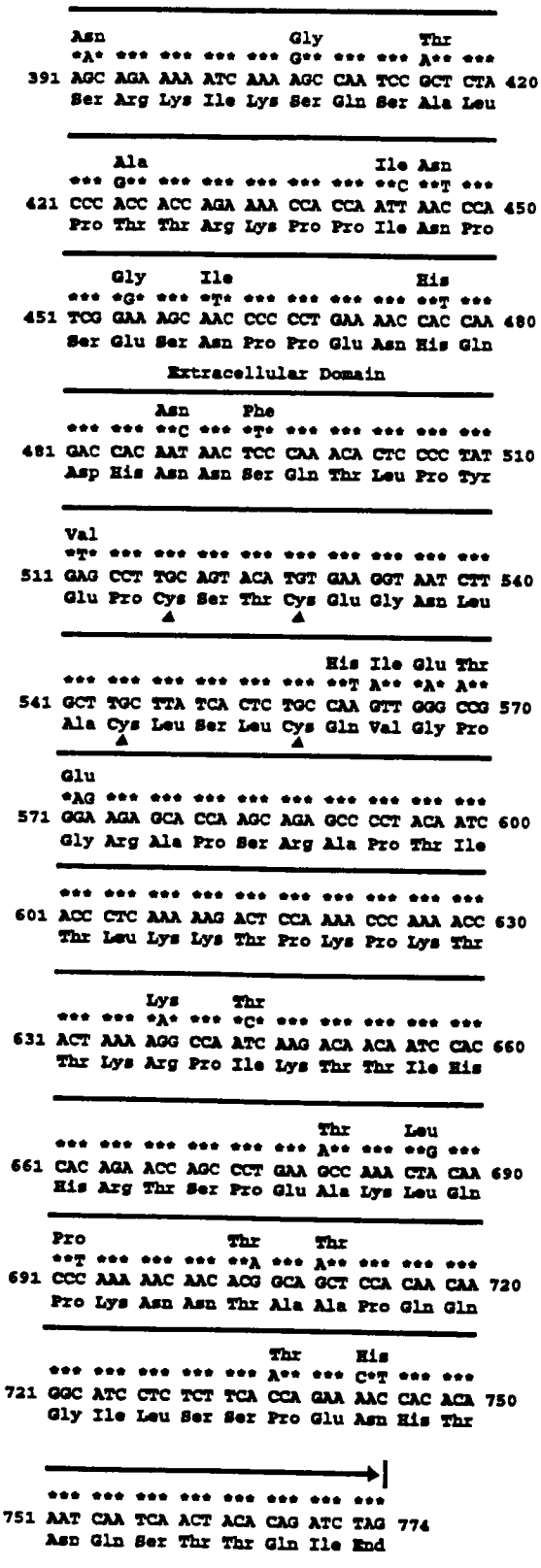
2

Figure 7. Nucleotide sequence of BRSV G glycoprotein gene cDNA. This schematic details the nucleotide sequence of the positive strand cDNA of the BRSV strain 375 G glycoprotein gene coding region. The 5' end of the gene is labelled "BRSV 375" and the nucleotides are displayed in groups of three with the corresponding deduced amino acid sequence depicted below. For comparison the nucleotide sequence of the BRSV 391-2 G glycoprotein gene (BRSV 391-2) is depicted above that of BRSV strain 375. Areas of homology are indicated by asterisks and nucleotide differences are indicated by letters. The corresponding amino acids encoded in areas of base differences are also indicated. Cytoplasmic, transmembrane, and extracellular protein domains are indicated by arrows. Four conserved cysteine residues in the extracellular domain are indicated by solid triangles.

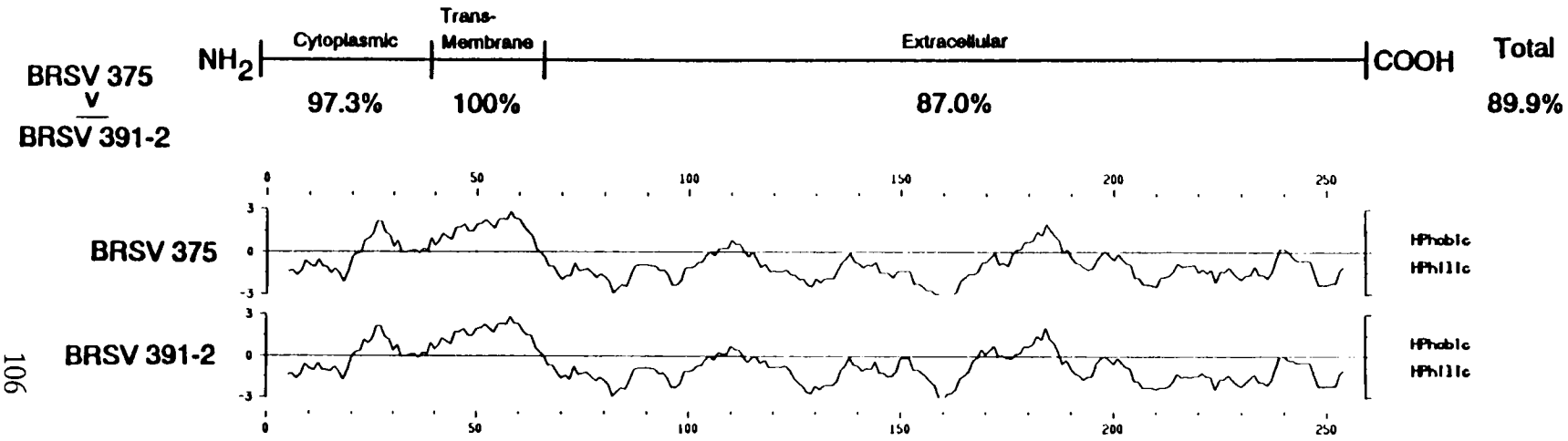

Cytoplasmic Domain
BRSV 391-2
BRSV 375
1 ATG TCC AAC CAT ACC CAT CAT CTT AAA TTC 30
Met Ser Asn His Thr His His Leu Lys Phe
Ala
31 AAG ACA TTA AAG AGG GCT TGG AAA CCG TCA 60
Lys Thr Leu Lys Arg Ala Trp Lys Pro Ser
Phe
61 AAA TAC TTC ATA GTA GGA TTA TCA TGT TTA 90
Lys Tyr Phe Ile Val Gly Leu Ser Cys Leu
91 TAT AAG TTC AAT TTA AAA TCC CTT GTC CAA 120
Tyr Lys Phe Asn Leu Lys Ser Leu Val Gln
Transmembrane Domain
121 ACG GCT TTG TCC ACC CTA GCA ATG ATA ACC 150
Thr Ala Leu Ser Thr Leu Ala Met Ile Thr
Ile
151 TTG ACA TCA CTC GTC ATA ACA GCC ATT ATT 180
Leu Thr Ser Leu Val Ile Thr Ala Ile Ile
Asn Ala
181 TAC ATT AGT GTG GGA AAG TCT AAA GCC AAG 210
Tyr Ile Ser Val Gly Lys Ser Lys Ala Lys
211 CCC ACA TCC AAA CCA ACC ATC CAA CAA ACA 240
Pro Thr Ser Lys Pro Thr Ile Gln Gln Thr
241 CAA CAG CCC CAA AAC CAT ACC TCA CCA TTT 270
Gln Gln Pro Gln Asn His Thr Ser Pro Phe
His
271 TTC ACA GAG AAC AAC TAC AAA TCA ACT CAC 300
Phe Thr Glu Asn Asn Tyr Lys Ser Thr His
Ser
301 ACA TCA ATT CAA AGC ACC ACA CTG TCT CAA 330
Thr Ser Ile Gln Ser Thr Thr Leu Ser Gln
Leu
Ile
331 CTA ATA AAC ATA GAC ACT ACT AGA GGA ACT 360
Leu Ile Asn Ile Asp Thr Thr Arg Gly Thr
Asn
361 ACA TAT GGT CAC TCA ACC GAC GAA ACC CAA 390
Thr Tyr Gly His Ser Thr Asp Glu Thr Gln


Asn Gly Thr
391 AGC AGA AAA ATC AAA AGC CAA TCC GCT CTA 420
Ser Arg Lys Ile Lys Ser Gln Ser Ala Leu
Ala Ile Asn
421 CCC ACC ACC AGA AAA CCA CCA ATT AAC CCA 450
Pro Thr Thr Arg Lys Pro Pro Ile Asn Pro
Gly Ile His
451 TCG GAA AGC AAC CCC CCT GAA AAC CAC CAA 480
Ser Glu Ser Asn Pro Pro Glu Asn His Gln
Extracellular Domain
Asn Phe
481 GAC CAC AAT AAC TCC CAA ACA CTC CCC TAT 510
Asp His Asn Asn Ser Gln Thr Leu Pro Tyr
Val
511 GAG CCT TGC AGT ACA TGT GAA GGT AAT CTT 540
Glu Pro Cys Ser Thr Cys Glu Gly Asn Leu
His Ile Glu Thr
541 GCT TGC TTA TCA CTC TGC CAA GTT GGG CCG 570
Ala Cys Leu Ser Leu Cys Gln Val Gly Pro
Glu
571 GGA AGA GCA CCA AGC AGA GCC CCT ACA ATC 600
Gly Arg Ala Pro Ser Arg Ala Pro Thr Ile
601 ACC CTC AAA AAG ACT CCA AAA CCC AAA ACC 630
Thr Leu Lys Lys Thr Pro Lys Pro Lys Thr
Lys Thr
631 ACT AAA AGG CCA ATC AAG ACA ACA ATC CAC 660
Thr Lys Arg Pro Ile Lys Thr Thr Ile His
Thr Leu
661 CAC AGA ACC AGC CCT GAA GCC AAA CTA CAA 690
His Arg Thr Ser Pro Glu Ala Lys Leu Gln
Pro Thr Thr
691 CCC AAA AAC AAC ACG GCA GCT CCA CAA CAA 720
Pro Lys Asn Asn Thr Ala Ala Pro Gln Gln
Thr His
721 GGC ATC CTC TCT TCA CCA GAA AAC CAC ACA 750
Gly Ile Leu Ser Ser Pro Glu Asn His Thr
751 AAT CAA TCA ACT ACA CAG ATC TAG 774
Asn Gln Ser Thr Thr Gln Ile End

The major open reading frame of our cDNA predicts a polypeptide of 257 amino acids (Figure 8) and a molecular mass of 28.4 kD; the major open reading frame of the BRSV strain 391-2 G glycoprotein gene also predicts 257 amino acids and a molecular mass of 28.6 kD. The deduced amino acid sequence of our clone shared 89.9% identity with the amino sequence from BRSV 391-2 (Figure 8). Amino acid differences between predicted amino acid sequences from our cDNA and BRSV strain 391-2 included 1, 0, and 25 differences in the proposed cytoplasmic, transmembrane, and extracellular domains respectively. These differences included a nonconserved sequence of five amino acids (amino acid residues 187-191) in the extracellular domain of our cDNA. However, hydropathy profiles of the predicted proteins from our cDNA and BRSV strain 391-2 were very similar (Figure 8).

Similar to the G glycoproteins of BRSV strain 391-2 and HRSV strains A2 and B8/60, our deduced G glycoprotein had a high serine and threonine content (25.6%): presumed to be the potential acceptor sites for O-linked oligosaccharides (14). The reported serine and threonine composition of BRSV strain 391-2 and HRSV strains A2 and 18537 were 25.7%, 30.6%, and 28.4%, respectively. The deduced amino acid sequences of both BRSV strain 391-2 and our clone predict 66 serine and threonine residues with 52 (79%) residing in the proposed extracellular domains. The two deduced amino acid sequences share 24 conserved serine residues and 36 conserved threonine residues. The nonconserved serine and threonine residues from BRSV strain 391-2 include six threonine residues whereas our clone had four nonconserved serine and two nonconserved threonine residues.

Figure 8. Amino acid identity between and protein hydropathy profiles of G glycoproteins from BRSV strains 391-2 and 375. This schematic depicts a linear representation of the BRSV G glycoprotein with the amino (NH_2) and carboxy (COOH) termini oriented to the left and right, respectively. Cytoplasmic, transmembrane, and extracellular protein domains are labelled and the corresponding amino acid identities are expressed as percent for each protein domain and total amino acid identity is displayed to the right. A comparison of the hydropathy profiles of each G glycoprotein is displayed beneath.


BRSV 375
v
BRSV 391-2
NH2
Cytoplasmic
Trans-
Membrane
Extracellular
COOH
Total
97.3%
100%
87.0%
89.9%
BRSV 375
BRSV 391-2
HPhobic
HPhilic
0
50
100
150
200
250
3
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-3

The deduced amino acid sequence of our clone also predicted a high proline content (8.9%) similar to that observed for G glycoproteins of BRSV 391-2 (7.8%) and HRSV strains A2 and 18537 (10% and 8.6%, respectively). Twenty of 23 proline residues predicted in our clone are conserved with the amino acid sequence of BRSV 391-2. Four cysteine residues were noted in the proposed extracellular domain of the predicted protein of our clone and their positions were exactly conserved with the postion of cysteine residues in other RSV G glycoproteins relative to the amino terminus of the protein.

CHAPTER 5

DISCUSSION

With our primers, BD-1 and BD-2, we amplified a 795 base pair cDNA fragment with total RNA from cells infected with BRSV strain 391-2 establishing that the primers could generate a specific cDNA with homologous virus RNA. We were also able to amplify a similar 795 base pair fragment with total RNA from BRSV strain 375 infected cells. This was not unexpected because we previously observed immunologic cross reactivity between the G glycoproteins of BRSV strains 391-2 and 375 in a polyclonal Western blot (Duncan, unpublished). The amplification of a cDNA fragment of similar size with total RNA from CRSV infected cells was unexpected because previous Western blot analysis indicated no immunologic cross reactivity between the G glycoproteins of BRSV and CRSV (Duncan, unpublished), although cross reactivity has been observed by others (22). This indicates that the 5' and 3' ends of the G glycoprotein genes of BRSV and CRSV share enough homology with our primers to permit primer annealing and PCR amplification. Non-specific PCR products generated using RNA from cells infected with CRSV and BRSV strains 391-2 and 375 might be explained by the following: primer concentration, magnesium concentration, polymerase enzyme concentration, and reaction parameters can all contribute to mispriming and the formation of non-specific background products. We optimized our PCR reaction profile to avoid non-specific products and found that by reducing primer concentration we could reduce the quantity of non-specific products, but we could not eliminate them. Significant secondary structure of our template RNA may have caused formation of shortened products by looping out of portions of RNA template

followed by polymerase read-through. Perkin-Elmer Cetus Corporation (Norwalk, CT) has described rTth thermostable polymerase that provides both reverse transcriptase and DNA polymerase functions. Our Taq polymerase might also have had reverse transcriptase activity, adding to the formation of non-specific products. We tested this possibility by, adding RNase between the reverse transcription step and the remainder of the thermocycling profile to avoid any possible Taq polymerase reverse transcriptase activity. This reduced the number and quantity of non-specific cDNA products, suggesting that Taq reverse transcriptase activity may have occurred (data not shown). Interestingly, PCR amplification, using the double stranded cDNA from recombinant lambda bacteriophage and pBluescript phagemid, generally yielded a single product of expected target length (Figures 5 and 6).

We were unable to amplify target cDNA using RNA from ORSV infected cells, suggesting that the ORSV G glycoprotein gene is divergent from BRSV. This is in agreement with our Western blot data where there was no serologic cross reactivity of the ORSV G glycoprotein with anti-BRSV or anti-HRSV sera, although there was cross reactivity of other ORSV proteins with the bovine and human sera. The first isolation of ORSV from sheep was confirmed by an indirect fluorescent antibody technique with sheep anti-BRSV serum (12). On a subsequent characterization study, the *in vitro* properties were limited to cytopathic effects of the virus on cell monolayers, indirect fluorescence, and virus neutralization (6). Certainly, it is reasonable to assume that ORSV has an analogous attachment protein, but its identity and properties remain obscure and undemonstrated.

The nucleotide sequence reported here is, to our knowledge, the second BRSV G glycoprotein gene to be characterized. We found 95.0% sequence identity

between our clone and the published G glycoprotein gene sequence of BRSV 391-2 (13).

We obtained our sequence data by performing at least three dideoxy chain termination reactions in both directions from one recombinant bacteriophage/ phagemid generated from one PCR reaction. First strand synthesis with reverse transcriptase and second strand synthesis with Taq polymerase could have introduced errors by polymerase infidelity. Additionally, repeated thermocycling could have contributed additional errors and/or amplified errors made early in the PCR amplification. The coding region of our G glycoprotein gene cDNA was identical in length and had a completely translatable open reading frame similar to that of BRSV strain 391-2 suggesting that our cDNA represented the full length G glycoprotein gene. Our primers, BD-1 and BD-2, were designed to extend 17 bases into the 5' and and 20 bases into the 3' end of the BRSV strain 391-2 G glycoprotein gene coding region. As a result, potential sequence differences in these areas could exist between the G glycoprotein genes from BRSV strains 391-2 and 375, yet would not be detected with our protocol.

The amino acid identity between deduced amino acid sequences from our cDNA and the G glycoprotein gene of BRSV strain 391-2 was 89.9% suggesting that these viruses belong to the same subgroup. Similarly, an amino acid identity of 94% was observed when two HRSV A subgroup virus G glycoproteins were compared (20). Also, the high serine and threonine content (25.6%) and an estimated molecular mass of 28.4 kD deduced from our cDNA were similar to that seen with the deduced amino acid sequence of BRSV strain 391-2. With Western blot analysis, the electrophoretic mobility of the BRSV strain 375 G glycoprotein was indicative of a molecular mass of 85-90 kD (Duncan, unpublished) suggesting that

extensive glycosylation probably contributes the remainder of the mature molecular mass. The many serine and threonine residues could serve as potential sites for extensive O-linked glycosylation in agreement with other reports (13,25).

Lerch et al. compared the deduced amino acid sequence of BRSV strain 391-2 G glycoprotein gene with conserved 13 amino acid sequences within the proposed extracellular domains of HRSV strains A2 and 18537 (13). This conserved region may be the receptor binding site of the G glycoprotein (9). They reported that BRSV strain 391-2 had six conserved amino acids in this region, of which two were cysteine residues. Two additional cysteine residues adjacent to this 13 amino acid region were also conserved between BRSV strain 391-2 and HRSV strains A2 and 18537 (13). Conserved cysteine residues may result in similar secondary structure among G glycoproteins and the differences in the conserved region could confer host specificity (13). We found conservation of only four amino acid residues in this 13 amino acid region when comparing the deduced amino acid sequence of our cDNA to the two HRSV strains, however all four cysteine residues were conserved. Our data suggest that BRSV strains 391-2 and 375 belong to the same BRSV subgroup. Lerch et al reported the possibility of two BRSV subgroups because the BRSV strain 391-2 G glycoprotein reacted with homologous bovine anti-BRSV strain 391-2 serum, but did not react with heterologous bovine anti-BRSV strain 127 serum (14). If this holds true, it will be interesting to see if the amino acid sequence of BRSV strain 127 G glycoprotein shares a small conserved region in the extracellular domain with amino acid sequences deduced from the G glycoprotein gene from BRSV strain 391-2 or our cDNA, analogous to the conserved sequence shared by HRSV subgroup A and B G glycoproteins. In addition, characterization of the G glycoprotein genes from CRSV and ORSV may also give further insight into host specificity and subgroup divergence of RSV G glycoproteins.

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PART 4

ANALYSIS OF RESPIRATORY SYNCYTIAL VIRUS (RSV) G GLYCOPROTEIN GENES BY RNase A MISMATCH CLEAVAGE

CHAPTER 1

ABSTRACT

The existence of more than one subgroup of bovine respiratory syncytial virus (BRSV) may have important implications in respiratory syncytial virus (RSV) disease pathogenesis and immunoprophylaxis. Furthermore, the molecular characterization of the G glycoprotein genes of different respiratory syncytial viruses may provide valuable information on molecular heterogeneity within RSV subgroups, species specificity, and host range of RSV strains. We have cloned a polymerase chain reaction-generated cDNA copy of the G glycoprotein gene from BRSV strain 375 into the Lambda ZAP bacteriophage vector. The purpose of this study was to examine RSV G glycoprotein gene divergence with a ribonuclease (RNase) mismatch technique. A radiolabelled anti-sense riboprobe to the BRSV 375 G glycoprotein gene was produced and cross-hybridized with total RNA extracted from cells infected with various RSV strains. Human RSV (HRSV) strains A2 and B8/60; BRSV strains 375, 391, FS1, NMK, 1143, 1144, and 16186; caprine RSV (CRSV); and ovine RSV (ORSV) were included in the study. RNA-RNA heteroduplexes were subjected to RNase digestion and products were resolved by polyacrylamide gel electrophoresis (PAGE) and autoradiography. Our results suggest that the BRSV strains in this study belong to the same subgroup, however genetic heterogeneity occurs among BRSV strains. Also, CRSV may be genetically more closely related to BRSV strains, whereas HRSV subgroups A and B and ORSV strains are more divergent from BRSV.

CHAPTER 2

INTRODUCTION

Respiratory syncytial virus (RSV) is an important cause of serious lower respiratory tract disease in both human infants and calves (8). It also has been isolated during respiratory disease outbreaks from sheep (9) and goats (20).

RSV is an enveloped virus with a negative sense, single stranded RNA genome (4). The genome has ten genes spread over approximately 15,000 nucleotides; each encodes a unique mRNA (4) transcribed from a single promoter at the 3' end (6). The mRNAs are translated into ten unique proteins, comprising eight structural and two nonstructural proteins (14). Of the eight structural proteins, two glycoproteins (G and F) constitute the major surface antigens of infected cells and virions (4). Both of these glycoproteins are important immunogenic proteins (7,17,22-24).

The antigenic variation of HRSV has been analyzed by characterizing the immunoreactivity of virus proteins with panels of monoclonal antibodies (1,16), providing a basis for division of HRSV strains into subgroups, designated subgroup A or B. Epitope differences between the two subgroups were found in four structural proteins including G, F, M, and N, but the only major differences resided in the G glycoprotein (16). A similar study of HRSV, BRSV, and CRSV strains also showed major antigenic differences between the G glycoproteins of the two human subgroups (18). Additionally, monoclonal antibodies against either human subgroup G glycoprotein did not react with BRSV or CRSV (18), suggesting that BRSV and CRSV belonged to an additional subgroup(s). Most but not all monoclonal antibodies against the other structural proteins of HRSV subgroup B

cross-reacted with the HRSV, BRSV, and CRSV (18). Other investigators have also compared the structural protein profiles of HRSV, BRSV, and CRSV (2,11,21) verifying that distinct antigenic differences exist between the G glycoproteins from HRSV and BRSV (11,21). Serologic cross-reactivity of G glycoproteins from BRSV and CRSV was detected (21). These studies collectively suggest that, in addition to the two human RSV subgroups, BRSV represents an additional distinct RSV subgroup that may be antigenically related to CRSV.

The existence of more than one BRSV subgroup is uncertain because few bovine isolates have been characterized. Lerch et al reported immunorecognition of BRSV strain 391-2 G glycoprotein by homologous anti-BRSV strain 391-2 serum, but not by heterologous anti-BRSV strain 127 serum, suggesting that like HRSV, BRSV also has antigenic subgroups (11). We examined, by Western blot analysis, the G glycoproteins of BRSV strains from various geographic sources and compared the immunorecognition of the G glycoproteins from BRSV, HRSV subgroups A and B, CRSV, and ORSV. Bovine anti-BRSV strain 375 serum reacted with the G glycoproteins of all BRSV strains, but not HRSV subgroups A or B, CRSV, or ORSV, whereas human anti-HRSV serum reacted with the G glycoprotein of the HRSV strain A2 only (Duncan, unpublished). We concluded that, with polyclonal serum immunodetection, we could not identify subgroup differences in the BRSV strains included in our study.

Although most of the molecular characterization of RSV has come from studies of HRSV, recently BRSV strain 391-2 proteins and mRNAs have been characterized and cDNA clones were derived from viral mRNAs (11). Comparison of the nucleotide sequences of the G glycoprotein genes from BRSV strain 391-2

and HRSV subgroups A and B demonstrate subgroup divergence of the BRSV G glycoprotein gene (10).

In a previous study using oligonucleotide primers based on the published BRSV 391-2 G glycoprotein sequence we amplified, cloned, and sequenced the G glycoprotein gene of BRSV strain 375 and demonstrated genetic heterogeneity between the two BRSV strains (Duncan, unpublished). The purpose of this study was to further characterize the G glycoprotein gene of several BRSV strains, CRSV, and ORSV. We produced an antisense radiolabelled riboprobe from the BRSV strain 375 G glycoprotein gene cDNA and did comparative analysis of HRSV strains A2 and B8/60, BRSV strains 375, 391-2, FS1-1, NMK7, 1143, 1144, and 16186, and the small ruminant strains CRSV and ORSV by RNase A mismatch cleavage.

CHAPTER 3

MATERIALS AND METHODS

Viruses and Cells

Eleven strains of RSV were obtained from various sources. HRSV strains A2 and B8/60 and BRSV strain 391-2 were provided by Dr. Gail Wertz (Department of Microbiology, The University of Alabama at Birmingham Medical School, Birmingham, AL). BRSV strains 375, FS1-1, NMK7; CRSV; and ORSV were provided by Dr. Howard Lehmkuhl (National Animal Disease Laboratory, Ames, IA). BRSV strains 1143, 1144, and 16186 were provided by Dr. James Collins (Colorado State University, Fort Collins, CO). Bovine turbinate cells (BTU-5) were provided by Dr. Kenny Brock (The Ohio Agricultural Research and Development Center, Wooster, OH). Viruses were grown in BTU-5 cells. The latter were grown in Dulbecco's Modified Eagle Medium (DMEM) (Hazelton Biologics, Inc., Lenexa, KS) supplemented with 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD). When approximately 50% of the cells in the monolayer had formed syncytia, they were suspended by scraping into the medium and dimethylsulfoxide (DMSO) was added to equal 5% of the total volume as a cryopreservative. One ml aliquots were stored at -70 C. Respiratory syncytial virus infection of BTU-5 cells was confirmed by direct immunofluorescence with a fluoroscein-labelled anti-RSV fusion (F) protein monoclonal antibody (Baxter Healthcare Corp., Bellevue, WA). Vero cells for cultivation of HRSV strains A2 and B8/60 were obtained from Dr. Leon Potgieter (The University of Tennessee, Knoxville, TN).

RNA Extraction

Bovine turbinate cells (BTU-5) grown in 75 cm^2 tissue culture flasks to 90% confluency, were infected with 0.5 ml of BRSV, CRSV, and ORSV virus preparations. Similarly, HRSV virus preparations were cultivated in Vero cells. The viruses were allowed to adsorb for 1-3 hours before the medium was replaced with fresh medium. When approximately 50% of the cells in each monolayer had formed syncytia, total RNA was extracted, using a technique modified from that described by Chomczynski and Sacchi (3). Virus-infected cells were scraped into the medium and centrifuged at 1000 g for 10 minutes to pellet the cells. The supernatant was poured off and the pellet was suspended in 1 ml of solution D (4M guanidinium thiocyanate; 25 mM sodium citrate, pH 7.0; 0.5% sarcosyl; 0.1 M 2-mercaptoethanol) in a 15 ml polypropylene tube. To this suspension, the following solutions were added sequentially with gentle mixing between each addition: 0.1 ml 2 M sodium acetate, pH 4.0; 1.0 ml water-saturated phenol; 0.2 ml chloroform-isoamyl alcohol, 49:1. The mixture was shaken vigorously for ten seconds and then placed on ice for fifteen minutes. The sample was divided into two equal volumes and placed in 1.7 ml microcentrifuge tubes and spun in a centrifuge at 10,000 g and 4 C for 20 minutes. The aqueous phase was transferred to a clean tube, 1 ml of isopropanol was added to it and held at -20 C for at least one hour to precipitate the RNA. The tube was the spun in a centrifuge at 10,000 g and 4 C for 20 minutes. The RNA pellet was dissolved in 0.3 ml of solution D and 1 ml of isopropanol was added and held at -20 C for at least one hour. The RNA was pelleted again by centrifugation at 10,000 g and 4 C for 20 minutes. The supernatant was removed

and the resultant pellet was washed with 70% ethanol. The pellet was dried in a vacuum concentrator (Savant Instruments, Inc., Farmingdale, NY) for approximately 5 minutes. The RNA was resuspended in 50 μl of diethyl pyrocarbonate-treated deionized water and the RNA was quantified by measuring absorbance at 260 nm in a spectrophotometer (Lambda 3B, UV/VIS, Perkin-Elmer Cetus, Norwalk, CT).

Preparation of Plasmid DNA for Transcription

Plasmid DNA for RNA transcription was prepared with a modification of the method described by Morelle (15). Two ml of super broth, in a sterile 15 ml polypropylene tube,(NaCl, 5 g/l; Bacto-yeast extract, 20 g/l; Tryptone, 35 g/l; and 5 M NaOH, 1 ml/l) was inoculated with XL1-Blue bacteria containing phagemid containing the G glycoprotein gene cDNA of BRSV strain 375, selected from a colony grown on LB/ampicillin agar and incubated overnight at 37 C. The broth culture was transferred to a 2.0 ml microfuge tube, spun in a centrifuge at 10,000 g for ten minutes, and the supernatant was poured off and the pellet saved. The cell pellet was resuspended in 200 μl of TSE lysis buffer (50 mM glucose; 25 mM Tris-HCl, pH 8.0; 10 mM EDTA) and lysozyme was added to a final concentration of 4 mg/ml and the mixture was incubated for five minutes at room temperature. Then, 400 μl of a 0.2 N NaOH, 1% sodium dodecyl sulfate (SDS) solution was added to the mixture, mixed gently, and the mixture was held on ice for five minutes. Three hundred μl of 7.5 M ammonium acetate was added to the mixture, mixed gently, and held on ice for ten minutes. The tube was then spun in a centrifuge at 10,000 g for

15 minutes and the supernatant was carefully transferred to a separate tube and isopropanol was added to equal 60% of the total volume and the tube was incubated at room temperature for ten minutes. Following centrifugation at 10,000 g for 30 minutes, the supernatant was removed and the DNA pellet was washed with 70% ethanol and dried in a vaccuum concentrator for five minutes.

Restriction Endonuclease Digestion of Plasmid DNA

The dried DNA pellet from the plasmid preparation was resuspended in 35 μl of distilled water and the following were added and mixed sequentially: 5.0 μl concentrated enzyme buffer C (International Biotechnologies, Inc., New Haven, CT), 2.5 μl EcoR 1 (16 U/μl) (International Biotechnologies, Inc., New Haven, CT), 2.5 μl Xho 1 (12 U/μl) (International Biotechnologies, Inc., New Haven, CT), and 5.0 μl of acetylated bovine serum albumin (BSA)(Promega, Madison, WI). The digestion mixture was incubated at 37 C for one hour. Following endonuclease digestion, the cDNA was extracted by adding an equal volume of phenol/chloroform. After centrifugation at 10,000 g for ten minutes, the cDNA in the aqueous phase was transferred to a clean tube and 0.1 volume of 3 M sodium acetate was added and the cDNA was precipitated by adding two volumes of 100% ethanol and placing the tube in -20 C overnight. The precitated cDNA was pelleted by centrifugation at 10,000 g for ten minutes, the supernatant removed, and the pellet was washed with 70% ethanol before drying in a vacuum concentrator for five minutes.

In Vitro Transcription of Radiolabelled Riboprobe

A radiolabelled antisense riboprobe was produced using a modification of the techniques described by Cristina (5) and Promega (Promega, Madison, WI). Plasmid DNA was linearized by endonuclease digestion with the enzyme EcoR 1 according to the procedure described previously. Approximately 5 μg of restriction endonuclease-digested plasmid was resuspended in 5 μl of diethylpyrocarbonate (DEPC)-treated deionized water. One μl of the digested plasmid was placed in a 0.6 μl microcentrifuge tube and the following were added and mixed sequentially: 4.0 μl transcription buffer concentrate (Promega, Madison, WI); 2.0 μl of 100 mM dithiothreitol (DTT); 20 units of RNasin (Promega, Madison, WI); 4.0 μl of nucleotide mixture (2.5 mM each of ATP, GTP, and UTP (Boehringer Manheim, Indianapolis, IN); 2.4 μl of 100 μM CTP (Boehringer Manheim, Indianapolis, IN); 50 Ci (5.0 μl) of [α-32P]-CTP (NEN Research Products, Boston, MA); 1.0 μl of bovine serum albumin (BSA, 2 mg/ml in water) (Fraction V, Sigma, St. Louis, MO); and 1.0 μl of T7 DNA polymerase (Promega, Madison, WI). After the transcription reaction mixture was incubated at 37 C for 60 minutes, RQ1 RNase-free DNase (Promega, Madison, WI) was added at a rate of 1 U/μg of DNA and the mixture then was incubated for an additional 15 minutes at 37 C. The mixture then was extracted with an equal volume of phenol/chloroform and the aqueous phase was mixed with 0.1 volumes of 3 M sodium acetate and 2.5 volumes of 100% ethanol and placed at -20 C overnight. The tube was spun at 10,000 g for ten minutes in a centrifuge; the precipitate was washed with 200 μl of 70% ethanol and the pellet was air dried. The pellet was resuspended in 150 μl of probe solution (10 mM Tris-HCl, pH 7.5; 1 mM EDTA; 0.1% SDS) and the radioactivity was

quantified in a model 1500 Tricarb Liquid Scintillation Analyzer (Packard Instrument Co., Downers Grove, IL).

Hybridization Reaction

The hybridization procedure was slightly modified from techniques described by Cristina (5) and Sambrook (19). Briefly, 2 x 10^5 cpm of radiolabelled probe was mixed with 30 μl of hybridization solution (80% deionized formamide; 0.4 M NaCl; 1 mM EDTA; 40 mM piperazine-N,N'-bis[2-ethanesulfonic acid] (PIPES), pH 6.7) that contained 30 μg of total RNA extracted from virus-infected BTU-5 cells. The mixture was heated to 85 C for five minutes and then immediately transferred to a 30 C water bath for two hours of incubation.

RNase Digestion Reaction

The RNase digestion reaction was modified from techniques described by Cristina (5). After hybridization, 300 μl of RNase solution (RNase A type III (Sigma), 40 μg/ml; RNase T1 (Boehringer Manheim), 2 μg/ml; NaCl, 300 mM; EDTA, 5mM; Tris-HCl, 10 mM, pH 7.5) was added and the mixture was incubated at 30 C for 30 minutes. Ten μl of 20% SDS and 5 μl of proteinase K (10 mg/ml) were added and the mixture was incubated at 37 C for 15 minutes. The mixture was extracted with an equal volume of phenol/chloroform and the aqueous phase was transferred to a new microcentrifuge tube and 11 μg of carrier tRNA (Sigma, St. Louis, MO) was added. One tenth volumes 3 M sodium acetate and 2.5 volumes of 100% ethanol were added and the mixture was held at -20 C overnight. On the

following day, the mixture was spun at 10,000 g for ten minutes and the pellet was washed twice with 200 μl of 70% ethanol and once with 100% ethanol and then air dried on the bench. The pellet was resuspended in 20 μl of gel loading buffer and heated to 85 C for two minutes and then placed on ice or stored at -20 C.

Polyacrylamide Gel Electrophoresis and Autoradiography

Ten μl samples of the RNase A digested hybridization products were loaded into the wells of an 8% polyacrylamide gel with 7M urea (Base Runner Nucleic Acid Sequencing Unit, International Biotechnologies, Inc., New Haven, CT) and electrophoretically separated at 2000 volts (constant voltage) for 3 hours. Following electrophoresis, the gel was removed and placed on blotting paper, wrapped in plastic wrap, and placed in a cassette with a piece of 35 by 43 cm Kodak X-OMAT AR film for autoradiographic exposure for five days at -70 C. The film was developed in a Kodak X-OMAT model 460 RA automatic film processor (Eastman Kodak Co., Rochester, NY).

CHAPTER 4

RESULTS

The results of our RNase A mismatch cleavage analysis are given in Figure 1. We observed complex band patterns of protected RNA:RNA duplex fragments from when our riboprobe was hybridized to RNA extracted from BTU-5 cells infected either with CRSV or any of the seven BRSV strains and then subjected to RNase A cleavage. The band patterns observed were very repeatable and separated the RSV strains into distinct groups. There were strong resemblences between the RNA duplex band patterns from BRSV strains 375, 1143, and 1144. BRSV strains 391-2 and NMK7 were similar to one another, whereas strains FS1-1 and 16186 were unique. Interestingly, the band pattern in BRSV strain FS1-1 and CRSV lanes resembled each other very closely. There were no bands evident in the control lanes (BTU-5 and Vero), or in lanes corresponding to the two HRSV strains (A2 and B8/60) or in the ORSV lane.

In order to estimate the genetic relatedness between pairs of different RSV strains with respect to our riboprobe derived from a cDNA of the BRSV strain 375 G glycoprotein gene, relatedness was expressed as a percentage of the fraction of bands shared by a pair of viruses, according to the formula: % = common bands in a pair of viruses divided by the total bands in the pair of viruses multiplied by two multiplied by 100. All distinctly discernable bands were counted in each lane as were discernable common bands in lane pairs. The data contained in Table 1 supports genetic heterogeneity among BRSV strains and their separation into groups. With respect to BRSV strain 375, BRSV strains 1143 and 1144 shared 88.2% and 85.7% common bands and comparison of the bands shared between

BRSV strains 1143 and 1144, 97.4% were common. CRSV and BRSV strain FS1 shared 85.4% common bands. While duplex RNA band patterns of BRSV strains 391 and NMK-7 look similar, especially the high molecular weight RNA duplex fragments at the top of Figure 1, percent common bands only equalled 30.8% and common bands shared between NMK-7 and CRSV or BRSV strains 375, FS1, 16186 were greater: 35.6, 45.0, 48.9, and 35.6, respectively.

Figure 1. Autoradiograph of RNase mismatch cleavage analysis. A radiolabelled antisense RNA probe generated from a cDNA copy of the BRSV strain 375 G glycoprotein gene was hybridized to total RNA extracted from cells either uninfected or infected with different respiratory syncytial virus strains. The lane designations are as follows: uninfected BTU-5 cells (1), uninfected Vero cells (2); Vero cells infected with HRSV strain A2 (3) or HRSV strain B8/60 (4); and BTU-5 cells infected with BRSV strain 375 (5), BRSV strain 391-2 (6), BRSV strain FS1-1 (7), BRSV strain NMK-7 (8), BRSV strain 1143 (9), BRSV strain 1144 (10), BRSV strain 16186 (11), CRSV (12), or ORSV.

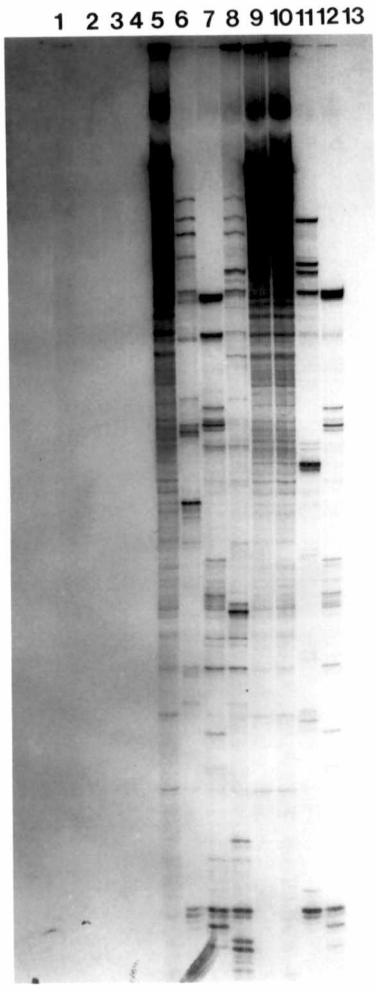
1 2 3 4 5 6 7 8 9 10 11 12 13

Table 1. **Common Bands (Percent) in the RNA Fragment Patterns of the BRSV strain 375G Glycoprotein Gene Riboprobe RNase A Mismatch Cleavage.**

	BTU-5	VERO	A_2	B8/60	375	391-2	FS1	NMK-7	1143	1144	16186	CRSV	ORSV
BTU-5	-	-	-	-	-	-	-	-	-	-	-	-	-
VERO		-	-	-	-	-	-	-	-	-	-	-	-
A_2			-	-	-	-	-	-	-	-	-	-	-
B8/60				-	-	-	-	-	-	-	-	-	-
375					100	25.7	42.7	45.0	88.2	85.7	24.2	34.3	-
391						100	19.0	30.8	15.4	21.5	27.5	25.0	-
FS1							100	48.9	28.6	24.4	29.3	85.4	-
NMK-7								100	29.9	21.4	35.6	35.6	-
1143									100	97.4	15.7	40.0	-
1144										100	15.1	38.7	-
16186											100	25.6	-
CRSV												100	-
ORSV													-

Note: Abbreviations used in the table are listed in Chapter 3, Materials and Methods. Percentages were calculated as % = (Common bands in a pair of viruses/Total bands in the pair of viruses) x 2 x 100.

CHAPTER 5

DISCUSSION

In this report we have used RNase mismatch cleavage to characterize the genetic heterogeneity of the G glycoprotein gene among various respiratory syncytial viruses. We did at least three hybridization and RNase digestion reactions using total RNA from cells infected with each virus strain and two separately transcribed probes. Results were readily reproducible and consistent.

We identified two genetic groups among the RSV strains tested based on the presence or absence of RNase A-protected bands. The BRSV and CRSV strains formed one group characterized by complex band patterns, whereas the HRSV and ORSV strains, which did not have RNase-protected bands, formed the other group. Theoretically, our antisense probe and G glycoprotein mRNA from BRSV strain 375 infected cells are complimentary and therfore one protected RNA duplex of probe length should be observed. However, a complex pattern of RNase-protected bands was observed when our radiolabelled riboprobe was hybridized to total RNA from the homologous virus (BRSV strain 375) and subsequently subjected RNase digestion. Similar results were observed by Cristina et al (5). They found complex band patterns when antisense probe made from HRSV was hybridized to total RNA from cells infected with homologous virus and subsequently subjected to RNase digestion. They also noted that more extensive RNase digestion led to more complex patterns and significantly increased the nonspecific background. Nevertheless, under identical cleavage conditions, their band patterns were always consistent and unique for each virus strain as were ours. During the transcription of radiolabelled antisense riboprobe, it is possible that not all transcripts were of full

length due to premature termination of transcription. Probes of varying lengths could have contributed to the complex band patterns. Other reasons for such complex band patterns might include genetic heterogeneity in viral RNA or errant and/or incomplete hybridization of probe to small sequences of homology in other parts of the G glycoprotein mRNA, elsewhere in other viral mRNAs, or to cellular RNA. However, although viral RNAs have been reported to hybridize to cellular ribosomal RNA (12,13) no bands were observed in control lanes that contained RNase digested heteroduplex RNA from uninfected BTU-5 or Vero cells.

The band patterns of the BRSV strains could be separated visually into several groups. BRSV strains 375, 1143, and 1144 had similar band patterns as did BRSV strains 391-2 and NMK7. The band patterns of BRSV strains FS1 and 16186 were not similar to any other BRSV pattern, although BRSV strain FS1 and CRSV closely resembled each other. The presence of complex patterns of protected bands with all BRSV strains and the CRSV strain in our study suggest significant sequence homology, however differences in band patterns among BRSV and CRSV strains supports genetic heterogeneity. The data in Table 1 supported the separation of those RSV strains that produced protected RNA duplex fragments into groups as evidenced by high percentage of common bands shared by BRSV strains 375, 1143, and 1144; and those shared by CRSV and FS1. However, a high percent of shared bands would be expected between BRSV strains 391 and NMK-7, but this was not the case when calculations were made. These two strains shared a significant number of high molecular size fragments, but there were frequent dissimilarities among lower molecular size fragments. These smaller fragments of BRSV strain NMK-7 were frequently common to other RSV strains and therefore the percentage of common bands shared by NMK-7 and other RSV strains often exceeded that

shared by BRSV strains NMK-7 and 391. We selected partial mismatch cleavage conditions similar to those described by Cristina et al (5), therefore, these conditions preclude absolute quantification of genetic differences between viral strains and Table 1 should only be considered as an index of differences between strains. Additionally, our calculations were made by counting distinctly discernable bands and discernable bands numbers could be easily influenced by autoradiographic exposure time. While the overall band patterns were readily reproducible, the combination of partial cleavage conditions and autoradiographic exposure time might have contributed to wide variations in the calculations included in Table 1.

RNase A mismatch cleavage was used by Cristina et al to analyze the genetic heterogeneity among HRSV isolates of both subgroups (A and B) (5). They compared genetic differences with antisense probes against five RSV structural proteins including P, N, 22K, F, and G derived from a HRSV subgroup A virus. The study showed that viruses of subgroups A and B formed two genetic groups based on very different patterns of RNase A-protected fragments, however, a surprising amount of genetic heterogeneity was present among viruses of the same subgroup. Sequence homologies of less than 86% generated simple patterns of small size bands (genes P, N, and F) or no bands at all (genes 22K and G). With respect to the G glycoprotein gene, the RNase A digestion of subgroup A heteroduplexes generated characteristic complex band patterns of protected fragments over a wide size range, whereas no bands were evident with subgroup B viruses. Because we observed complex band patterns with CRSV and all BRSV strains, we believe that these strains most likely share nucleotide sequence homologies of greater than 86%. In agreement with Cristina et al, we recently cloned and sequenced G glycoprotein gene cDNA from BRSV strain 375 and found 95.0% identity between the nucleotide

sequence of our cDNA and a published sequence from BRSV strain 391-2 (Duncan, unpublished). Both BRSV strains were included in our study and each had complex band patterns.

Lerch et al suggested the possibility of more than one BRSV subgroup when they compared the immunoreactivity of the BRSV strain 391-2 G glycoprotein with homologous (anti-BRSV strain 391-2) and heterologous (anti-BRSV strain 127) sera (11). However, we were unable to detect subgroup differences with a Western blot analysis study of the G glycoprotein from the same seven BRSV strains included here (Duncan, unpublished). Our RNase mismatch cleavage analysis data of these same seven BRSV strains also suggests that these viruses belong to the same subgroup. Admittedly, we would like to include BRSV strain 127 in future studies to verify if BRSV strain 127 does represent a BRSV strain subgroup unique from the seven BRSV strains that we have characterized.

Our data also suggests a close genetic relationship between BRSV and CRSV strains. Complex patterns of RNase A-protected bands indicate significant homology between BRSV and CRSV. In a previous study, we amplified a cDNA fragment (approximately 795 base pairs) from RNA extracted from CRSV-infected cells with oligonucleotide primers (BD-1 and BD-2) designed from sequence data of the BRSV strain 391-2 G glycoprotein gene suggesting there was significant homology between the 5' and 3' ends of the CRSV and BRSV 391-2 G glycoprotein genes (Duncan, unpublished). However, we also have examined the G glycoproteins from BRSV and CRSV strains by Western blot analysis and did not detect antigenic relatedness between BRSV and CRSV G glycoproteins (Duncan, unpublished). In a comparative Western blot analysis Trudel et al found serologic cross reactivity between the G glycoproteins of BRSV and CRSV suggesting

antigenic relatedness (21). We can not readily explain the differences between these observations, but differences in blotting procedure, viruses, or in antiserum may explain the discrepancy.

As expected, we did not find RNase-protected RNA duplexes in the HRSV A2 and B8/60 lanes. We estimated, based on sequence data from HRSV strains A2 and 18537 and from our BRSV strain 375 G glycoprotein cDNA, that approximately 48% nucleotide sequence homology existed between our riboprobe and the G glycoprotein genes of HRSV strains A2 and 18537. Complete degradation of the HRSV A2 and B8/60 RNA heteroduplexes in our study suggests that BRSV should be classified as a subgroup separate from HRSV.

Although ORSV was recovered from a ruminant, no RNase-protected RNA duplexes were seen in the ORSV lane. Attempts to amplify the ORSV G glycoprotein gene by polymerase chain reaction using oligonucleotide primers BD-1 and BD-2, designed from sequence data from a BRSV G glycoprotein gene (10), were unsucessful (Duncan, unpublished). These observations suggest significant divergence of the ORSV G glycoprotein gene from BRSV. Therefore, ORSV may represent a second ruminant RSV subgroup. To our knowledge, the G glycoprotein and G glycoprotein gene of ORSV have not been characterized.

In summary, our data suggests that the BRSV strains included in our study belong to the same BRSV subgroup and CRSV shares significant homology with the BRSV strains. Also, the HRSV subgroups A and B are divergent from BRSV and CRSV. Finally, ORSV is also divergent from BRSV and CRSV. We are currently cloning and sequencing the ORSV G glycoprotein. We feel that sequence data from various RSV strains, especially BRSV strain 127 and ORSV, will be very important in lending further insight into the divergence of respiratory syncytial viruses and

provide for future studies of RSV immunopathogenesis, immunoprophylaxis, and host range specificity.

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PART 5

SUMMARY

CHAPTER 1

SUMMARY OF PARTS 2-4

We studied strain divergence of respiratory syncytial viruses (RSV) of ruminants, an integral component in the complex etiology of bovine respiratory disease (7). Two human RSV (HRSV) strains, representing HRSV subtypes A and B; seven bovine RSV (BRSV) strains from various geographic sources; and the two small ruminant strains caprine RSV (CRSV) and ovine RSV (ORSV) were included in the study.

Although, a report by Lerch et al suggested that more than one subtype of BRSV exists (5), the various strains of BRSV, CRSV, and ORSV have not been well characterized. HRSV strains have been subdivided into two subgroups based on distinct antigenic differences in an important immunogenic surface glycoprotein, G (1,6). These two subgroups cocirculate in respiratory disease outbreaks in humans (2) and it is unclear if these subgroup differences are important in disease pathogenesis. Characterization of ruminant RSV subtypes is warranted because of implications on pathogenesis and prophylaxis of infections by these viruses. The study of ruminant RSV may also provide an animal model to study HRSV infection and immunity, replacing more controversial HRSV animal models. BRSV infection in calves closely approximates the respiratory disease seen in human infants (3). If more than one BRSV subgroup exists, their study could determine the importance of subgroup differences in disease pathogenesis and immunoprophylaxis. The bovine model would also be advantageous to study passive immunity, because antibody levels could be manipulated by regulation of colostrum intake.

The purpose of this work was to characterize the G glycoproteins and G glycoprotein genes of ruminant RSV strains. We obtained seven BRSV strains, CRSV, ORSV, and two HRSV strains (subtypes A and B) and characterized their G glycoproteins and G glycoprotein genes by three methods. Initially, we detected antigenic differences between G glycoproteins from the various RSV strains with an immunoassay using serum. We determined that bovine anti-BRSV strain 375 serum reacted with the G glycoproteins from all BRSV strains included in our study, but did not react with the G glycoproteins of the two HRSV strains, CRSV, or ORSV. In contrast, anti-HRSV serum reacted with the G glycoprotein from HRSV strain A2 only. These results suggested that the BRSV strains in our study belonged to the same subgroup, distinctly different from the HRSV subgroups A and B. Also, the results suggested that CRSV and ORSV belonged to an additional subgroup(s), distinct from BRSV and HRSV.

In the second phase, we designed and used oligonucleotide primers defining the bounderies of the BRSV G glycoprotein gene, based on a published sequence, to amplify cDNA from various ruminant RSV genomes. We cloned the amplified cDNA of BRSV strain 375 into a suitable vector system to determine the nucleotide sequence of the BRSV cDNA. Templates for PCR amplification in total RNA from cells infected with two BRSV strains (391-2 or 375) and CRSV were identified, but total RNA from ORSV-infected cells was refractory. These results suggested that CRSV and BRSV share nucleotide sequence homology, at least at the 5' and 3' ends of the G glycoprotein gene. Nucleotide sequence of BRSV 375 G glycoprotein gene cDNA was compared to the published sequence of BRSV strain 391-2 (4). We found 95.0% nucleotide sequence identity and a 89.9% amino acid sequence identity of their predicted proteins encoded by these two genes. Four cysteine

residues in the proposed extracellular domain of the proteins were conserved and most of the serine and threonine residues were conserved also. These results support inclusion of these two BRSV strains in the same subgroup.

Finally, we produced a radiolabelled riboprobe from our cDNA clone and compared various RSV strains by RNase mismatch cleavage. Complex band patterns observed with all BRSV and CRSV strains suggested that BRSV and CRSV strains, included in the study, belong to the same RSV subgroup although distinct genetic heterogeneity among strains is evident. Inclusion of CRSV and BRSV in the same RSV subgroup is in agreement with our earlier polymerase chain reaction analysis. As expected, the data was also consistent with previous reports suggesting that BRSV and HRSV A and HRSV B belonged to three distinct subgroups (4,5). Our data supports that ORSV likely belongs to an additional RSV subgroup distinct from CRSV, HRSV, and the BRSV strains tested. However, we have not definitively established that ORSV differs from HRSV B8/60.

We concluded that the BRSV strains tested were closely related genetically. However, some heterogeneity existed among these BRSV strains. Although Lerch et al suggested that BRSV strains 391-2 and 127 belonged to different BRSV subgroups, we were unable to test BRSV strain 127 in our studies. Sequence data from BRSV strain 127 and its comparison with BRSV strains 375 and 391-2, will be important in establishing the existence of more than one BRSV subtype. Caprine RSV may be genetically related to BRSV, but we were unable to confirm the G glycoprotein antigenic relatedness between these viruses in Western blot analysis. Sequence analysis of CRSV G glycoprotein gene should explain this discrepancy. Additionally, ORSV may belong to a distinct RSV subgroup. Molecular characterization of the G glycoprotein gene from this virus will add to the

understanding of G glycoprotein heterogeneity and its relationship to host range specificity. Since the ORSV produces disease in calves experimentally, it is possible that the ORSV strain may actually represent a BRSV subtype unique from the other BRSV strains tested in our laboratory. As RSV G glycoprotein genes are cloned and characterized, they may constitute important ingredients in recombinant vaccines since glycoprotein G is one of the major immunogens of this virus. Recombinant viruses and protein subunit vaccines may provide effective RSV immunoprophylaxis and aid control of bovine respiratory disease.

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VITA

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