Bovine respiratory syncytial virus fusion protein gene: sequence analysis of cDNA and expression using a baculovirus vector

S. Roy Himes and Laurel J. Gershwin*

University of California, Davis, Agricultural Experiment Station, School of Veterinary Medicine, Department of Veterinary Microbiology and Immunology, Davis, California 95616-8738, U.S.A.

The nucleotide sequence of bovine respiratory syncytial virus (RSV), ATCC strain A51908 fusion (F) glycoprotein gene cDNA was determined. The amino acid sequence deduced was then compared to those of two different isolates of bovine RSV, strains RB 94 and 391-2, and the A and B subtypes of human RSV, strains 18537 and A2. The bovine RSV F protein is highly conserved between the three isolates, A51908 has 97% amino acid identity to RB 94, and 99% identity to 391-2. The F proteins of both the A and B types of human RSV are 81% identical to that of A51908. The cDNA clone was expressed using a baculovirus vector and the expressed recombinant F protein produced in SF9 cells was characterized by Western blot analysis. The recombinant F protein was post-translationally cleaved into the active form and reacted with serum from bovine RSV-infected calves.

Bovine respiratory syncytial virus (RSV) is a negative strand RNA virus of the family Paramyxoviridae and a major cause of respiratory tract disease in cattle. Infection with bovine RSV produces a mild to severe pneumonia, predominantly in calves below 6 months of age. A similar pathology is observed in children infected with a closely related virus, human RSV (Stott & Taylor, 1985).

Human RSV has been characterized extensively at the molecular level. The fusion (F) envelope glycoprotein is highly immunogenic, well conserved between virus strains and elicits the highest cytotoxic T lymphocyte response (Olmsted et al., 1986; Pemberton et al., 1987). Antibody to the F protein can neutralize infectivity of extracellular virus and prevent cell fusion (Walsh et al., 1987). Molecular characterization of bovine RSV is beginning and cDNA clones of both the attachment (G) envelope glycoprotein and the F glycoprotein genes from strain 391-2 have been expressed using vaccinia virus vectors (Lerch et al., 1990, 1991). Comparison of the F protein amino acid sequences deduced for bovine and human RSVs has shown that the F protein gene is highly conserved, whereas the G protein gene is not (Walravens et al., 1990; Lerch et al., 1990).

The F protein is synthesized as a 70K F₀ precursor that is post-translationally cleaved into F₁ (48K) and F₂ (23K) subunits linked by disulphide bonds (Gruber & Levine, 1983). Cleavage by cell protease(s) is necessary for fusion activity.

Vaccine-related problems have been reported for both bovine and human RSV in individuals vaccinated with inactivated or live attenuated virus vaccines (Kim et al., 1969; Wright et al., 1982; Kimman et al., 1989). These problems have led to research into the development of a subunit vaccine, possibly to enhance the immune response to critical virus proteins and limit the hypersensitivity encountered with previous vaccines. The fact that the F protein is conserved between strains of RSV and its importance in generating a protective immune response make it a likely component of this type of vaccine. A recombinant approach to F protein production was favoured because production of an effective and commercially feasible vaccine would require large amounts of viral protein. The baculovirus expression system allows the production of large amounts of recombinant protein in insect cells (Matsuura et al., 1987), whereas high levels of expression are not possible with the vaccinia virus system. Since insect cells utilize many of the protein processing systems of higher eukaryotic cells, post-translational modification of recombinant protein is possible (Cochran et al., 1987). An F gene cDNA clone of human RSV has been expressed using a baculovirus vector, and cotton rats vaccinated with the recombinant protein are protected from live virus challenge. The recombinant F protein is able to induce antibodies which neutralized both virus and cell fusion (Wathen et al., 1989).

cDNA cloning of bovine RSV F gene sequences was performed to obtain a complete copy of the coding region for use in sequence analysis and subsequent expression of
Fig. 1. The nucleotide and deduced amino acid sequences of bovine RSV strain A51908, and comparison with the deduced amino acid sequences of bovine RSV strains 391-2 and RB 94, and human RSV strains 18537 (type B) and A2 (type A). Possible sites for N-linked glycosylation are boxed. The hydrophobic region at the N terminus of F1 is underlined.
recombinant protein. In this communication, we present the nucleotide sequence and deduced amino acid sequence of the bovine RSV F gene from ATCC laboratory strain A51908, and a comparison with the deduced amino acid sequence from existing F gene cDNA clones of bovine and human RSV. We also describe the expression of the A51908 F gene cDNA clone using a baculovirus vector and the characterization of the recombinant F protein produced in SF9 cells.

To produce a λ gt10 cDNA library, cDNA was prepared from the mRNA of bovine RSV (A51908)-infected, actinomycin D-treated (1 μg/ml) Madin Darby bovine kidney cells using the method of Gubler (1987). Clones containing F gene sequences were isolated by in situ hybridization of λ phage plaques. The probe used was a cDNA clone of the human RSV F gene from strain A2 (Johnson & Collins, 1988). 32P-labelled probe was first hybridized to total RNA from bovine RSV-infected cells to determine conditions for cross-hybridization to the bovine RSV F gene and the time of maximum production of F gene mRNA. All cross-hybridizations were done overnight at 37 °C in 35% formamide with 5× saline-sodium phosphate-EDTA (SSPE) buffer. The inserts from potential F gene recombinants were electrophoresed on 1% agarose gels to exclude short sequences. Three phage clones were isolated, containing inserts of approximately the size of a complete copy of the F gene (>1800 bp). These inserts were subcloned into pTZ 18U for sequence analysis.

DNA sequencing of clone F1A revealed a full-length insert containing the entire coding region of the bovine RSV F gene. Nucleotide sequencing was done according to the dideoxynucleotide chain termination method of Sanger et al. (1977) on an alkali-denatured pTZF1A plasmid using the Sequenase DNA sequencing kit (United States Biochemical).

Comparison of the amino acid sequence deduced from the A51908 clone to those deduced from F gene cDNA from two different isolates, 391-2 (Lerch et al., 1990) and RB 94 (Walravens et al., 1990), indicated that the F protein is highly conserved within the bovine RSV subtype (Fig. 1). The percentage identity between A51908 and 391-2 and RB 94 was 99% and 97%. This high degree of sequence identity between A51908 and 391-2 suggests they are the same or a closely related strain. The bovine RSV A51908 F gene cDNA clone has virtually the same level of amino acid identity with F gene cDNA clones of the B subtype strain of human RSV, 18537, as with that of the A subtype strain, A2 (Johnson & Collins, 1988) (Fig. 1). Although the position of amino acid changes varies somewhat, the overall identity with both strains is 81%. The F1 subunit has a greater degree of conservation between bovine and human RSV, 89% as compared to 68% for F2.

The sequence analysis showed that cDNA clone F1A of the bovine RSV F gene contained an intact coding region with very little 5' leader sequence, making it suitable for expression in the baculovirus system. The F1A insert from the original phage clone was subcloned into the baculovirus expression plasmid pVL 1393 (Invitrogen). Caesium chloride gradient-purified plasmid DNA and DNA from the wild-type baculovirus, Autographa californica nuclear polyhedrosis virus (Invitrogen), was cotransfected into Spodoptera frugiperda (SF9) cells by the calcium phosphate precipitation method of Burand et al. (1980). Virus was harvested 4 days post-transfection and recombinant baculovirus containing the bovine RSV F gene insert was isolated by in situ hybridization of virus plaques to 32P-labelled F1A DNA, according to the method of Villareal & Berg (1977). DNA purified from cells infected with plaque-isolated recombinant baculovirus was transferred to nylon for dot blot hybridization. DNA from cells infected with either an unrelated recombinant baculovirus, producing β-galactosidase, or wild-type baculovirus (Invitrogen) was used as a negative control. All hybridizations were done at 42 °C in 50% formamide with 5× SSPE.

Recombinant baculovirus was then screened for expression of F protein by immunoblotting of infected cell lysates on nitrocellulose. Anti-RSV antibody-positive bovine serum from calf 28, vaccinated and infected with bovine RSV strain A51908, was used as the primary antibody in dot blots, with horseradish peroxidase-conjugated rabbit anti-bovine IgG (H & L) (Jackson ImmunoResearch) as the secondary antibody. Lysates from cells infected with wild-type baculovirus or recombinant β-galactosidase-expressing virus were used as negative controls.

Cells infected with the recombinant baculovirus were then examined by an indirect immunofluorescence assay using a commercial rabbit anti-RSV antibody, shown to cross-react with the bovine RSV F protein (Dako Laboratories), and a fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (Zymed) was used for visualization. Recombinant β-galactosidase-expressing baculovirus-infected cells were used as a negative control. A high level of fluorescence was present in cells infected with the recombinant F protein-expressing baculovirus; only background fluorescence was present with the recombinant β-galactosidase-expressing baculovirus-infected negative control. This fluorescence indicates a high level of F protein expression which appears to be present on the surface of infected cells (Fig. 2).

To characterize the recombinant F protein produced in the insect cells, a Western blot was performed on cell lysates infected with the recombinant F protein-expressing baculovirus. Cell lysates infected with the recombinant protein.
Short communication

Fig. 2. Indirect immunofluorescence assay of acetone-fixed SF9 cells infected with recombinant F protein-expressing baculovirus (a), or with recombinant β-galactosidase-expressing baculovirus as a negative control (b). The exposure time of the negative control was increased to visualize cells. Bar marker represents 25 μm.

Fig. 3. Western blot of infected cell lysates electrophoresed under reducing and non-reducing conditions. Reducing conditions: SF9 cells infected with recombinant F protein-expressing baculovirus (lane 1), wild-type baculovirus, negative control (lane 2), recombinant β-galactosidase-expressing baculovirus, negative control (lane 3) or polyethylene glycol-precipitated bovine RSV (strain A51908) grown on BK cells (lane 4). Lanes 5, 6 and 7 contain samples similar to those in lanes 1 to 3, electrophoresed under non-reducing conditions.

The profile of bands on the Western blot under reducing and non-reducing conditions corresponded to the expected Mr’s of the whole bovine RSV F protein, F₀, and the F₁ and F₂ subunits (Fig. 3) (Lerch et al., 1991). There were three bands on the Western blot of the recombinant F protein-expressing baculovirus-infected cell lysate denatured under reducing conditions. A band was present at approximately 68,000, corresponding to the size of the whole F protein containing both the F₂ and F₁ subunits. This indicated that a fraction of the recombinant F protein produced by the insect cells is not cleaved by proteases during post-translational processing. The same result was found when recombinant human RSV F protein was expressed in SF9 cells and may verify that the insect cell protease recognizes the F₂–F₁ cleavage site only poorly. Another band was present at approximately 46,000, corresponding to the size of the F₁ peptide. A faint band is present at a position equivalent to approximately 4000 below the 46,000 band. This band may result from the presence of a second cleavage site within the F₁ peptide recognized by an insect cell protease because a similar result was found with the baculovirus-expressed human RSV F protein (Wathen et al., 1989). A final band occurs at 20,000, corresponding to the approximate Mr of the F₂ peptide.

Non-reduced baculovirus-expressed F protein samples produced a single band at 68,000, the Mr of the F₀ protein. An unidentified non-specific band at 96,000 to 97,000 was present in the β-galactosidase-expressing baculovirus-infected negative control. No bands were present in the wild-type baculovirus-infected control.

The results of the immunological analysis showed that a portion of the recombinant F protein is cleaved into its active form in insect cells and is recognized by antiserum from cattle immunized with native protein. The availability of this recombinant F protein will permit studies to analyse the immune response to the F protein only. Large scale production and purification of recombinant protein has the potential to provide antigen for a subunit vaccine, which could then be experimentally tested in
cattle. However, because the F protein is embedded in the SF9 cell membrane it will be necessary to develop a method of purification from cell proteins before quantitative estimates of the amount of protein expressed per cell can be made. The advantage of the bovine system is that the vaccine trials could be held in the natural host of bovine RSV. Another advantage is that cattle are a suitable size for multiple sample collection, which would allow an in-depth analysis of the immune response. One would then be able to investigate fully the response to different types of vaccines in an attempt to find one that best produces a protective response to bovine RSV.

We would like to thank Dr Peter Collins for providing the RSV strain A2 F cDNA clone used for screening, and Grant Bullock and Marta Camacho for excellent technical assistance. This research was supported by USDA grant 89-34116-6878.

References


(Received 7 October 1991; Accepted 10 February 1992)