Bovine respiratory syncytial virus nucleocapsid protein: mRNA sequence analysis and expression from recombinant vaccinia virus vectors

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The nucleotide sequence of the mRNA encoding the nucleocapsid (N) protein of bovine respiratory syncytial (BRS) virus, strain 391-2, was determined. Recombinant vectors containing a cDNA of the complete N gene were constructed, and expression of the N protein in eukaryotic cells was demonstrated using two different vector systems. The BRS virus N mRNA was 1197 nucleotides in length, exclusive of poly(A), and had a single major open reading frame that encoded a polypeptide of 391 amino acids with a calculated Mr of 42-6K. The nucleotide and amino acid sequences of the BRS virus N gene were compared to those of human respiratory syncytial (HRS) virus strains A2 and 18537, and to BRS virus strain A51908. The level of nucleic acid identity between the N mRNA of BRS virus 391-2 and both HRS virus subtypes was 80 to 81 %, whereas the identity between the two BRS virus strains was 97 %. A 93 to 94 % level of identity was observed between the deduced amino acid sequences of the N protein of BRS virus 391-2 and the corresponding sequences of the two HRS virus strains. The two BRS virus N proteins differed in amino acid sequence at only three positions. Recombinant BRS virus N protein was expressed using two different vector systems: in cells from a plasmid using the vaccinia virus/T7 polymerase expression system or from a recombinant vaccinia virus. N proteins synthesized by the two vector systems migrated with an electrophoretic mobility identical to that of authentic BRS virus N protein, and were precipitated by anti-BRS virus antibodies.

Bovine respiratory syncytial (BRS) virus is an important cause of serious respiratory illness in the cattle industry (Bohlender et al., 1982; Stott & Taylor, 1985; Stott et al., 1980). The disease caused in calves is similar in pathogenicity to that caused by human respiratory syncytial (HRS) virus in children. However, the host range of BRS virus differs from that of HRS virus in that the former is narrower. BRS virus, like HRS virus, is an enveloped, single-stranded, negative-sense RNA virus (Lerch et al., 1989). The BRS virus genome is transcribed to yield 10 mRNAs which code for 10 proteins that correspond closely in size to the HRS virus proteins (Lerch et al., 1989; Cash et al., 1977; Westenbrink et al., 1989). The BRS virus genes encoding the two major surface glycoproteins, the attachment glycoprotein (G) and the fusion glycoprotein (F), have been sequenced and their protein products expressed from recombinant vaccinia viruses (VV) (Lerch et al., 1990, 1991). These recombinant viruses are currently being evaluated for their ability to elicit protective immune responses in cattle. The HRS virus nucleocapsid (N) protein is a target for cytotoxic T lymphocyte (CTL) responses in humans and in mice, and stimulates a protective immune response in mice (King et al., 1987; Bangham et al., 1986). However, recent work has shown differences in the CTL response to individual HRS virus antigens when examined in mice as compared to humans (Askonas et al., 1989). For this reason, evaluation of the role of individual BRS virus antigens in establishing immune responses in the natural host, cattle, is important both for understanding the role of BRS virus proteins in infection and for evaluating the CTL responses in small animal models as compared to the natural host. To examine the role of the N protein in both humoral and cell-mediated immunity to BRS virus we constructed recombinant eukaryotic expression vectors. The nucleotide sequence of the BRS virus N gene was determined and expression of the N protein in cells was characterized using two types of recombinant expression vectors.

The BRS virus 391-2 strain used in this report was isolated from cattle in North Carolina, U.S.A. during the winter of 1984 to 1985. cDNA clones corresponding to the BRS virus genes were generated and characterized as reported previously (Lerch et al., 1989). The nucleotide sequence of the N protein mRNA was determined from

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Fig. 1. Nucleotide sequence of the BRS virus strain 391-2 N mRNA. The nucleotide sequence of BRS virus N mRNA as determined from cDNA clones and primer extension on mRNA is shown. Comparison with the HRS virus strains A2, 18537 and BRS virus strain A51908 are also shown. Only the nucleotides that differ are shown. The dots above the sequence are spaced every 10 nucleotides.

five independent cDNA clones. The nucleotide sequences of the two largest clones, J4 and G33, were obtained by subcloning cDNA fragments isolated by digestion with restriction enzymes (G33) or by sequencing a series of nested deletions (J4 and G33), which were created using the ExoIII–ExoVII unidirectional deletion technique (Ozkaynak & Putney, 1987). Nucleotide sequences were obtained by dideoxynucleotide chain termination as described (Lim & Pene, 1988; Tabor & Richardson, 1987) using M13 forward and reverse sequencing primers (New England Biolabs). The largest clone, G33, was missing only the first 10 nucleotides from the 5' end of the mRNA. J4, the second largest clone, lacked the first 20 nucleotides, including the start codon, from the 5' end of the mRNA. Three other clones sequenced ranged from 400 to 500 nucleotides in length and were located at the 3' end of the mRNA. The 5' end of the BRS virus 391-2 N mRNA was determined directly by extension of an oligonucleotide primer on mRNA isolated from BRS virus 391-2-infected cells. The nucleotide sequence of this oligonucleotide primer, complementary to bases 74 to 91 of the N mRNA, was selected from the sequence obtained from the cDNA clones. Variation was observed at only one position in the N mRNA sequence. In clone J4, the nucleotide at position 472 was a C instead of the T shown in the consensus sequence (Fig. 1).

The nucleotide and deduced amino acid sequences of the BRS virus 391-2 strain were compared to other known RS virus N protein nucleotide and amino acid sequences using the University of Wisconsin Genetics Computer Group software package (Devereux et al., 1984). The consensus nucleotide sequence, exclusive of the 5'-terminal nucleotide, is shown in Fig. 1 along with
the conserved gene start sequence (5' GGGCAAAAT 3') shared by all but two of the 10 HRS virus mRNAs (Johnson & Collins, 1988). The BRS virus N mRNA contained the consensus sequence found at the 3' end of all HRS virus mRNAs [AUA/AA(N)]-4-poly(A)] (Collins et al., 1986). The BRS virus 391-2 F and G mRNAs also shared these conserved gene start and gene end sequences (Lerch et al., 1990, 1991).

The nucleotide and deduced amino acid sequences of the BRS virus N gene were compared to those of HRS virus strain A2 (a subgroup A virus) (Collins et al., 1985) and HRS virus strain 18537 (a subgroup B virus) (Johnson & Collins, 1989), and to those of another BRS virus, strain A51908 (Samal et al., 1991). The BRS virus N mRNA contained 1197 nucleotides, excluding a poly(A) tail. The single major open reading frame started with an initiation codon beginning at nucleotide 16 and extended to a termination codon beginning at nucleotide 1189. The polypeptide encoded by the N mRNA was 391 amino acids in length with a calculated M₀ of 42.6K which is consistent with the apparent M₀ of 43K determined by SDS-PAGE (Cash et al., 1977; Lerch et al., 1989).

The sequence at the 5' end of the mRNA conformed to the conserved gene start sequence (5' GGGCAAAAT 3') shared by all but two of the 10 HRS virus mRNAs (Johnson & Collins, 1988). The BRS virus N mRNA contained the consensus sequence found at the 5' end of all HRS virus mRNAs [AUA/AA(N)]-4-poly(A)] (Collins et al., 1986). The BRS virus 391-2 F and G mRNAs also shared these conserved gene start and gene end sequences (Lerch et al., 1990, 1991).

The level of identity between the four different N mRNA sequences that were compared (Fig. 1) is very high, corresponding to previous observations that the N gene is one of the more highly conserved genes of HRS virus (Johnson & Collins, 1989). Previous sequence comparisons of BRS virus 391-2 F and G mRNAs to the corresponding HRS virus mRNAs demonstrated levels of nucleotide identities of 71 or 72% between the BRS virus 391-2 F mRNA sequence and the HRS virus 18537 and A2 F mRNA sequences, respectively, and levels of nucleotide identities of 51 or 52% between the BRS virus 391-2 G mRNA sequence and the HRS virus 18537 and A2 G mRNA sequences (Lerch et al., 1990, 1991).

The level of identity between the four different N protein amino acid sequences deduced from the sequence of BRS virus strain 391-2 N mRNA is shown. Comparison with HRS virus strains A2, 18537 and BRS virus strain A51908 is also shown. The dots above the sequence are spaced every 10 amino acids.

**Table 1. Percentage nucleotide and deduced amino acid sequence identities between the N genes of BRS viruses and HRS viruses**

<table>
<thead>
<tr>
<th></th>
<th>391-2 versus A51908*</th>
<th>391-2 versus A2†</th>
<th>391-2 versus 18537‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall mRNA</td>
<td>97.4</td>
<td>80.4</td>
<td>81.2</td>
</tr>
<tr>
<td>Amino acid</td>
<td>99.2</td>
<td>93.6</td>
<td>93.4</td>
</tr>
</tbody>
</table>

* Comparison of BRS virus isolate 391-2 and BRS virus isolate A51908 (Samal et al., 1991).
† Comparison of BRS virus isolate 391-2 and HRS subgroup A virus (A2) (Collins et al., 1985).
‡ Comparison of BRS virus isolate 391-2 and HRS subgroup B virus (18537) (Johnson & Collins, 1989).
the highly conserved nature of this protein. Most of the nucleotide differences observed did not result in amino acid changes. Of interest is the region between amino acids 220 and 371 (Fig. 2), which shows variation at only two residues. This level of amino acid sequence identity is consistent with the antigenic cross-reactivity observed between the BRS virus and HRS virus N proteins (Lerch et al., 1989).

To examine the expression of the BRS virus N protein from cloned DNA, clone G33, containing the entire N protein-coding region, was used to produce BRS virus N mRNA for N protein synthesis using the VV/T7 RNA polymerase expression system described by Fuerst et al. (1986). Clone G33, containing the N gene downstream of the T7 RNA polymerase promoter in pIBI76, was transfected using lipofectin into bovine turbinate (BT) cells that had been infected with the recombinant VV vTF7-3 which expresses T7 polymerase (Fuerst et al., 1986; Pattnaik & Wertz, 1990; Lefkowitz et al., 1990). Parallel cultures were also infected with BRS virus 391-2 for comparison. After 5 h the transfected cells were labelled with [3H]leucine for 2 h, followed by harvest and immunoprecipitation of proteins using M117PO3 (anti-BRS virus calf serum) as previously described (Lerch et al., 1989; Wertz et al., 1985). Proteins from the BRS virus strain 391-2-infected cells were harvested at 25 h post-infection. Proteins were analysed by SDS-PAGE (Laemmli, 1970) and detected by fluorography (Chamberlain, 1979) (Fig. 3). As shown in Fig. 3, the expression of this clone produced a protein having an electrophoretic mobility of approximately 42K that was immunoprecipitated by anti-BRS virus serum and that comigrated with the BRS virus N protein from 391-2 virus-infected cells (Fig. 3, lane 3), confirming that clone G33 contained a complete copy of the BRS virus 391-2 N mRNA.

A VV recombinant expressing BRS virus N protein was constructed also in order to examine the immune response to the BRS virus N protein in animals. This was done by first placing clone G33 into the unique Smal site of the VV plasmid vector, pAB 191 (Stott et al., 1986) which contains the VV 7.5K promoter inserted in the thymidine kinase (tk) gene. The orientation of G33 was determined by restriction enzyme digestion. The N gene and associated 7.5K promoter were transferred into the tk gene of the Copenhagen strain of VV by homologous recombination. Recombinant viruses (tk-) were selected by three rounds of plaque purification in medium containing 5-bromo-2'-deoxyuridine and their identity was confirmed by hybridization of recombinant virus DNA with a probe specific for the BRS virus N gene (Lerch et al., 1990, 1991). Expression of the N protein from the recombinant virus designated vvNbov was compared with that of N protein synthesized in BRS virus-infected BT cells and cells infected with wild-type Copenhagen VV. The infected cells were labelled with [35S]methionine for 2 h after which the proteins were harvested, immunoprecipitated and analysed by SDS-PAGE as described previously (Lerch et al., 1989), except that the immunoprecipitations were done with a mixture of two monoclonal antibodies, MAB 7 and MAB 15, to the HRS virus N protein. In Fig. 4, lanes 1 and 2 show uninfected and BRS virus 391-2-infected cells, respectively, and lane 3 shows that infection with the VV N recombinant yields the expression of a protein with an electrophoretic mobility identical to BRS virus N protein. No protein of this mobility was immunoprecipitated from the mock-infected or VV-infected controls.
the recombinant protein was reduced in comparison to that synthesized in the BRS virus 391-2-infected cells and in comparison to other recombinant VV vectors expressing BRS virus proteins (Lerch et al., 1990, 1991). This result may be explained by the presence of a consensus VV transcription termination signal, TTTTTAT (Rohrman et al., 1986), located at position 911 of the BRS virus N mRNA sequence. The production of less than full-length recombinant N gene transcripts would result in less N protein synthesized per infected cell. A similar termination signal was observed in the HRS virus A2 NmRNA sequence, which resulted in the expression of truncated recombinant N mRNA (King et al., 1987).

This recombinant VV expressing the N protein of BRS virus 391-2 will be used to study the role of BRS virus N protein in eliciting a protective immune response against BRS virus challenge in cattle.

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