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Analysis of a hypervariable region
in the 3' non-coding end
of the infectious bronchitis virus genome

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(Received 13 August 1992; revision received 3 December 1992; accepted 4 December 1992)

Summary

Previous studies on infectious bronchitis virus (IBV) cDNA have identified a region of about 184 bases in the 3' non-coding terminus of both the U.S. prototype strain (Beaudette) and a Japanese strain (KB8523), that was not present in an antigenically closely related U.S. strain, Massachusetts (Mass) 41 (Boursnell et al., 1985; Sutou et al., 1988). In order to investigate the origin and function of this region and its occurrence in nature, the cDNA sequences of the 3' non-coding regions of three additional strains of IBV, Gray, Arkansas (Ark) 99 and Holland (Holl) 52, were determined and compared to the sequences of the Beaudette, KB8523 and Mass41 strains. Not only was this U-rich sequence absent from the 3' non-coding region of the Mass41 strain, it was also highly variable, especially in comparison to the highly conserved 3' non coding region downstream of this sequence. Computer analyses of the sequences adjacent to this hypervariable region (HVR) showed that the 3' end of the IBV genome was highly conserved downstream of this region, with 94.3 to 97.8% similarity. However, the similarities for the HVR ranged from 53.2% between Holl52 and Ark99, to 92.8% between

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Beaudette and Gray. The flanking sequences were not only conserved but these sequences upstream and downstream of the HVR also formed mirrored images.

Infections bronchitis virus; Hypervariable region; Sequencing; 3' non-coding region

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Avian infectious bronchitis virus (IBV) is a highly contagious pathogen of chickens with many strains varying widely in virulence, serotype, and pathotype (Darbyshire et al., 1979; Hopkins, 1974). For example, Beaudette is a highly attenuated strain of IBV, Holl52 is a vaccine strain, Gray and a Japanese strain (KB8523) are both known to be nephropathogenic but have also been associated with respiratory disease, and Ark99 and Mass41 are generally thought to result in severe respiratory disease in the absence of gross kidney lesions (Beaudette and Hudson, 1937; Cumming, 1963; Darbyshire et al., 1979; Johnson and Marquardt, 1986; Hungherr et al., 1956; Sutou et al., 1988; Winterfield and Hitchener, 1962). ArkDPI strains (embryonating chicken egg passages 11 and 75) belong to the same serogroup as Ark99 (Sneed et al., 1989).

IBV was the first coronavirus to be completely sequenced and is the prototype of the family Coronaviridae (Boursnell et al., 1987). The IBV genome is 27.6kb in length and consists of single-stranded RNA of positive polarity with a polyA tail (Schochetman et al., 1977). Viral replication takes place under the control of a viral RNA-dependent RNA polymerase produced by translation of the genomic RNA. The polymerase presumably binds to the 3' end of the genomic RNA to initiate transcription, resulting in the production of negative sense RNA, either full length or subgenomic length, from which mRNAs can be transcribed (Boursnell et al., 1987, Sethna et al., 1989).

Previous studies on IBV cDNA for the 3' non-coding terminus of both a U.S. strain (Beaudette) and a Japanese strain (KB8523) identified a region of 184 bp with a higher U content that the remainder of the genomic RNA (Boursnell et al., 1985; Sutou et al., 1988). This region, however, was not present in Mass41, an antigenically closely related strain. The 3' non-coding regions of additional strains were cloned and sequenced in order to investigate functional importance and to determine the extent of this U-rich sequence within the IBV group of *Coronaviruses*. A similar U-rich region of 184 bases was found in the sequence of the Ark99 genome immediately downstream of the nucleocapsid gene where it had been described in the Mass-related strains. A 40-base synthetic oligonucleotide was synthesized that was complementary to the middle of this Ark99 U-rich region, and was labelled at the 3' end using ^{32}P-dCTP and terminal deoxy transferase (Sambrook et al., 1989). Four IBV RNAs, Mass41, Holl52 and 2 Arkansas isolates (ArkDPI-11 and ArkDPI-75) were applied to and baked on a nitrocellulose membrane. The RNA was applied in four-fold dilutions, starting with 1 μg, and dot blot hybridisation was performed at 42°C overnight in the presence of formamide (Sambrook et al., 1989).

Holl52

ArkDPI-75 ● ● ● ● ●

ArkDPI-11 ● ● ● ● ●

Mass41
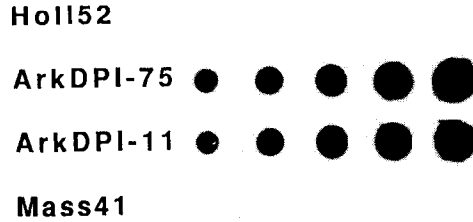
Fig. 1. Dot blot of RNA from 4 strains of IBV. RNA was immobilized onto a nitrocellulose membrane and probed with a ^{32}P-labelled synthetic oligonucleotide complementary to a portion of the U-rich region in the 3′ non-coding region of Ark99.

Hybridization studies with this Ark99-derived probe detected similar sequences in genomic RNA from the ArkDPI-11 and ArkDPI-75 strains, but not in Mass41 or Holl52 RNA, indicating, as expected, the presence of a similar U-rich sequence in ArkDPI-11 and ArkDPI-75 (Fig. 1). However, this probe also did not hybridize to Holl52, suggesting either that the strain did not contain this region or that it was too dissimilar to be recognized by the Ark99-derived probe. The cDNA sequences of two additional strains, Gray and Holl52, were then examined, and a comparable region was found in both.

Since the 3′ non-coding region of the IBV genome should be important in the recognition and/or binding of polymerase, we examined the sequences of this region of the genome. The viral isolates used in this study, previously described by Sneed et al. (1989), were propagated at 37°C for 36–48 h following allantoic sac inoculation into 11-day-old specific pathogen-free embryonating chicken eggs. The eggs were then refrigerated at 4°C for 5 h, and the allantoic fluid collected and clarified by centrifugation at 10K rpm for 20 min. Virus was precipitated overnight with 8% polyethylene-glycol, 2.33% NaCl, and concentrated by centrifugation at 10K rpm for 20 min. Virus pellets were reconstituted in 1/50 vol. 20 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.4 and banded on a 30–50% glycerol/potassium tartrate gradient for 2 h at 28K rpm. After concentrating by ultracentrifugation at 28K rpm for 1 h, virus pellets were resuspended in 0.1 M NaOAc, pH 5.2, and the virions were disrupted with 1 mg/ml proteinase K and 1% SDS at 37°C for 1 h. The RNA was extracted first with phenol and then with chloroform/isoamyl alcohol followed by ethanol precipitation (Wang et al., 1988). Presence of IBV genomic RNA was confirmed by electrophoresis of a sample of the RNA on a 1% agarose gel and the remaining RNA was reprecipitated with ethanol.

Purified RNA was pelleted and dried in a Savant vacuum drier. RNA was resuspended in 7 μl deionized H$_2$O (dH$_2$O) and denatured in 6 mM methyl mercuric hydroxide for 10 min at room temperature. First strand cDNA synthesis was carried out using 5 units of AMV reverse transcriptase at 42°C for 2 h in the presence of 14 mM 2-mercaptoethanol (2ME), 50 mM Tris pH 8.3, 50 mM KCl, 8 mM MgCl$_2$, 0.8 mm dNTP, 20 μg/ml of 3′ end primer (5′ GGATCCGCTC-TAACTCTATACTAGCCTAT 3′) and 20 units of RNasin (Promega, Madison, WI). Following phenol extraction and ethanol precipitation of the RNA/cDNA

hybrid, the RNA was eliminated by exposure to highly alkaline conditions at 37°C for 3 h. The cDNA was then neutralized, concentrated by ethanol precipitation, and amplified using the polymerase chain reaction (PCR). PCR was performed with the same 3' end primer as above and a primer from the upstream intergenic region (5'GAATTCCCGCGTGTACCTCTCTAGTA 3') in the presence of 4 mM dNTP, $1 \times Taq$ buffer (1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8) and 0.5 μl Taq polymerase (Perkin Elmer, Norwalk, CT). The PCR was performed with a primary denaturation step at 95°C for 3 min, annealing at 50°C for 30 s, extension at 72°C for 1 min, and subsequent denaturation steps at 95°C for 1 min. A total of 30 cycles were used with a final extension step of 3 min at 72°C. Resulting PCR products were cloned into the pCR1000 vector, utilizing the single T overhang at the insertion site (Invitrogen, San Diego, CA) and sequenced using the Sequenase rit (USB, Cleveland, OH). Nucleotide sequences were processed using the GCG Seqed program and compared with the Gap and Bestfit programs. Sequences were aligned using Pileup and predicted secondary structures of the 3' non-coding region were derived from Fold and Squiggles.

The nucleotide sequences of the 3' non-coding regions of the Ark99, Gray and Holl52 strains of IBV were compared with the published sequences for Mass41, Beaudette and KB8523 (Boursnell et al., 1985; Sutou et al., 1988). Fig. 2 shows the alignment of these sequences and demonstrates the high degree of similarity among the strains.

However, the U-rich region upstream of the 3' non-coding region, that was absent from Mass41, was found in Holl52, as well as Gray and Ark99, and was described as the 3' hypervariable region (HVR) because of the extent of its variation among strains. This HVR ranged from 184 (Gray) to 196 (Holl52) nucleotides in length and the nucleotide sequence homologies among the strains containing this sequence ranged from 53.2% to 92.8%, with the Holl42 strain showing the greatest divergence (Table 1). The greater variability of the Holl52 strain compared to the other strains in this region immediately downstream of the nucleocapsid gene would explain why this region was not detected with the Ark99 probe (Fig. 1). In comparison, the non-coding region downstream of this HVR was highly conserved, ranging from 94.3% to 97.5% identity (Table 2). Fig. 3 shows a plot of the similarity of the nucleotide sequences of the 3' non-coding regions of all 6 strains of IBV. The HVR is clearly seen immediately after the nucleocapsid gene and can be compared to the remaining 3' non-coding region which has very high similarity scores. This HVR could be considered either an insertion or a deletion since it is not present in Mass41. However, the higher U content in comparison to the rest of the IBV genome suggests that it is of exogenous origin (Boursnell et al., 1985). The fact that it has at least 53.2% identity among various strains suggests a common ancestry for this sequence.

If the IBV genome acquired the HVR as exogenous RNA, this region could be a site for recombination. Sequences upstream and downstream of the HVR were analyzed to identify any distinctive patterns which would suggest that this region is of functional significance, such as being involved in recombination. Not only are the sequences flanking the 3' HVR highly conserved, the 5' flanking sequence also

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                                                                               19
Ark       GGATTCAGCT TTAGGTGAGA ATGAACTTG  A.G.......  ........TAA
Gray      AGATTCTYGCT TTAGGTGAGA ATGAACTCTG AGG.......  ........TAA
Beaudette GGATGCAGCT CTAGGAGAGA ATGAACTTTG A.G.......  ........TAA
KB8523    TGACTCAGCA CTTGGTGAAA ATGAACTTTG A.T.......  ........TAA
Mass41    GGATTCAGCT CTAGGAGAGA ATGAACTTTG AGT.......  .........
Hol152    GGATTCAGCA CTTGGAGAGA ATGAGTTGTA AAGCTAGATT  TCCAACTTAA
                                                    **  *

                                                                               69
Ark       CATAATGGAC CTTGCTGCATT CTTTGGTAC.  ........AT TTTGTTAGCC
Gray      CATAATGGAC CTTGCTGCATT TTTTGGTAC.  ........AT TTTGTTAGCC
Beaudette CATAATGGAC CTGTTG..TT  TCCTGGTAC.  ........AT TTTGTTAAAC
KB8523    CATAATGGAC TTGCTGCACT  TTCTCTGGCC.  ........AT TTCTTTGTGT
Mass41
Hol152    CATCANGGAC GTGCGTATGC  TGTITTTCGC  TACTATAGAC ATCCTAGCAT

                                                                              119
Ark       ATTATTTTCTG TGCTTTCCTA TCAATCATTA CAGGCCATTGA TTGTGATCAT
Gray      ACTATTTTTG  TGCTTTCCTA TCAATTATTA CAGGCCATTGA TTGTGATTAT
Beaudette ACTATTTCTG  TGCTTTCCTA TCAATTATTA CAGGCCATTGA TTGTGATTAT
KB8523    TTTGTTTTTG  TAGATTTCTC ACAATTATTA TTGG.AATGA  TTGTGATTAT
Mass41
Hol152    ATAAITTTTT  GCTAITTGA  TGGTTAITTA CAGGTGAAGA  TTGTATGTAT

                                                                              169
Ark       GTGCAATATT TAAGTTCTT  TTGGTTGCTT TTTGCTTGTT GTATTGTTGC
Gray      GTGCAATATT TAAGCTTCTT  TTGGTTGCTT TTTGCTTGTT GTGTTGTTGC
Beaudette GTTCAATACT TAAGCTTCTT  TTGGGTTGCTT TTTGCTTGTT GTATTGTTGC
KB8523    GTGCAGTATC TTAGTTGGTT TAATTATCTT  GTGTTCAGTTT GTGTTCTTTC
Mass41
Hol152    TTGTTGTACA CTCGTATGGT CTATATTAGG TTTTCTTGTAG TTGTTATTAG

                                                                              219
Ark       TGTGCTTTTT ATTAITGTGA TTCTCCATTAG TTTGTTTTAT CGTAGAAGTT
Gray      TGTGCTTTTT ATTAITTGTGA TTCTC...AG TTTGCTTTAT CGTAGAAGTT
Beaudette TGTGCTTTTT AITGTTGTGA TTCTCATTAG TTTGCTTTAT CGTAGAAATT
KB8523    TGTGCTTATT GTTTTTATGC TGCTTATTC  TGCTTTATAT CGTAGAAGTT
Mass41
Hol152    TGTTGTTCTT GTCCTTACTC TACTGTTCTC TTTTCTTTAT TTAGGAGTAT

                                                                              269
Ark       CAATAGTAAG AGTTAAGGAA GATAGGCATG TAGCTTGATT ACCTACACTGT
Gray      CAATAGTAAG AGTTAAGGAA GATAGGCATG TAGCTT.AGC ACCTACACTGT
Beaudette CAATAGTAAG AGTTAAGGAA GATAGGCATG TAGCTTGATT ACCTACACTGT
KB8523    ..TATTGAA  AGTTAAGGAA GATAGGCATG TAGCTTGATT ACCTACACTGT
Mass41    CAATAGTAAG AGTTAAGGAA GATAGGCATG TAGTTTGATT ACCTACACTGT
Hol152    CAATA...AG AATCAAGGAA GATAGGCATG TAGTTTGATT ACCTACACTGT

                                                                              319
Ark       CTATCGCCAG GGAAATGTCT AATCTGTCTA CTTAGTAGCC TGGAAACGAA
Gray      CTATCGCCAG GGAAATGTCT AATCTGTCTA CTTAGTAGCC TGGAAACGAA
Beaudette GTATCGCCAG GGAAATGTCT AATCTGTCTA CTTAGTAGCC TGGAAACGAA
KB8523    CTATCGCCAG GGAAATGTCT AATCTGTCTA CTTAGTAGCC TGGAAATGAA
Mass41    CTATCGCCAG GGAAATGTCT AATTTGTCTA CTTAGTAGCC TGGAAACGAA
Hol152    CTATCGCCAG GGAAATGTCT AATCTGTCTA CTTAGTAGCC TGGAAACGAA

                                                                              369
Ark       CGGTAGACCC TTAGATTTTA ATTTAGTTTA ATTTTTAGTT TAGTTTAAGT
Gray      CGGTAGACCC TTAGATTTTA ATTTAGTTTA ATTTTTAGTT TAGTTTAAGT
Beaudette CCGTAGACCC TTAGATTTTA ATTTAGTTTA ATTTTTAGTT TAGTTTAAGT
KB8523    CGGTAGACCC TTAGATTTTA ATTTAGTTTA ATTTTTAGTT TAGTTTAAGT
Mass41    CGGTAGACCC TTAGATTTTA ATTTAGTTTA ATTTTTAGTT TAGTTTAAGT
Hol152    CGGTAGACCC TTAGATTTTA ATTTAGTTTA ATTTTTAGTT TAGTTTAAGT

                                                                              419
Ark       TAGTTTAGAG TAGGTATAAA GATGCCAGTG CCGAGGCCAC GCGGAGTACG
Gray      TAGTTTAGAG TAGGTATAAA GATGCCAGTG CCAGGGCCAC GCGGAGTACG
Beaudette TAGTTTAGAG TAGGTATATA GATGCCAGTG CCGGGGCCAC GCGGAGTACG
KB8523    TAGTTTAGAG TAGGTATAAA GATGCCAGTG CCGGGGCCAC GCGGAGTACG
Mass41    TAGTTTAGAG TAGGTATAAA GATGCCAGTG CCGGGGCCAC GCGGAGTACG
Hol152    TAGTTTAGAG TAGGTATAAA GAAGCCAGTG CCGGGGCCAC GCGGAGTACG

                                                                              469
Ark       ATCGAGGGTA CAGCACTAGG ACGCCCACTA GGGGAAGAGC TAAATTTTAG
Gray      ATCGAGGGTA CAGCACCAGG ACGCCCCATTA GGGGAAGAGC TAAATTTTAG
Beaudette ATCGAGGGTA CAGCACTAGG ACGCCCCATTA GGGGAAGAGC TAAATTTTAG
KB8523    ATCGAGGGTA CAGCACTAGG ACGCCCCACTA GGGCAAGAGC TAAATTTTAG
Mass41    ACCGAGGGTA CAGCACTAGG ACGCCCATTA  GGGGAAGAGC TAAATTTTAG
Hol152    ATCGAGGGTA CAGCACTAGG ACGCCCACTA  GAGGAAGAGC TAAATTTTAG

                                                                              519
Ark       TTTAAGTTAA GTTTAATTTA CTAAGTATAG TTAAAATTTA TAGGCTAGTA
Gray      TTTAAGTTAA GTTTAATTGG CTAAGTATAG TTAAAATTTA TAGGCTAGTA
Beaudette TTTAAGTTAA GTTTAATTGG CTAAGTATAG TTAAAATTTA TAGGCTAGTA
KB8523    TTTAAGTTAA GTTTAATTGG CTAATGTATAG TTAAAATTTA TAGGCTAGTA
Mass41    TTTAAGTTAA GTTTAATTGG CTATGTATAG TTAAAATTTA TAGGCTAGTA
Hol152    TTTAAGTTAA GTTTAATTGG CTAAGTATAG TTAAAATTTA TAGGCTAGTA

                                                      532
Ark       TAGAGTTAGA GC.
Gray      TAGAGTTAGA GC.
Beaudette TAGAGTTAGA GC.
KB8523    TAGAGTTAGA GCA
Mass41    TAGAGTTAGA GC.
Hol152    TAGAGTTAGA GC.
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Fig. 2. Nucleotide sequence comparison of the 3' non-coding region of 6 strains of IBV. Nucleotides are numbered from the stop codon for the nucleocapsid gene which is indicated by ***. The hypervariable region starts immediately after the stop codon and continues to base 213. Conserved bases in this region are underlined.

TABLE 1

Percent homologies of the 3′ HVR

	Ark99	Gray	Beau	Holl52	KB8523
Ark99	100	91.8	91.8	53.2	68.6
Gray	91.8	100	92.8	57.9	67.6
Beau	91.8	92.8	100	57.1	66.7
Holl52	53.2	57.9	57.1	100	60.0
KB8523	68.6	67.7	66.7	60.0	100

TABLE 2

Percent homologies of the 3′ non-coding region of IBV

	Ark99	Gray	Beau	Holl52	KB8523	Mass41
Ark99	100	97.2	97.5	94.7	97.2	96.3
Gray	97.2	100	97.8	94.3	96.8	96.6
Beau	97.5	97.8	100	94.7	97.2	97.5
Holl52	94.7	94.3	94.7	100	95.3	94.3
KB8523	97.2	96.8	97.2	95.3	100	94.7
Mass41	96.3	96.6	97.5	94.3	94.7	100

resembles the cutting site (5′ TTAA 3′) of retrovius DNA and transposons Tn10 and Tn3 (Shoemaker et al., 1980). It was of special interest that a unique pattern, that may be of functional importance, was observed in the oligonucleotides flanking the HVR. Several nucleotides downstream of this 3′ HVR were mirrored in the sequences upstream. That is, the same sequences were seen in reverse orientation on either side of the 3′ HVR. An example can be seen in the Gray strain where the sequence TGAGAATG appears 5′ of the HVR and the sequence
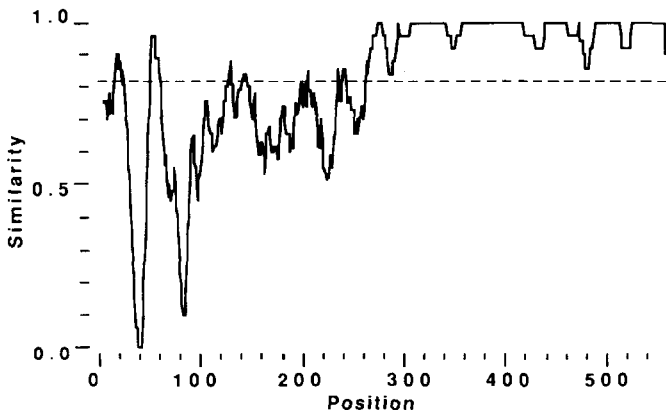

Fig. 3. Similarity plot of the 3′ non-coding region of six strains of IBV. This plot starts from the end of the nucleocapsid gene and covers the 3′ HVR and conserved 3′ end.

TABLE 3

Mirrored sequences flanking the hypervariable region

| * | indicates the position of the insert. Sequences mirrored on both sides of hypervariable region are underlined.

ARK9	TGAGAATGAACTTTGAA	*	AGTTCAATAGTAAGAGT
GRAY	TGAGAATGAACTCTGAGG	*	AGTTCAATAGTAAGAGT
BEAU	AGAGAATGAACTTTGAG	*	AATTCAATAGTAAGAGTT
KB8523	GAAAATGAACTTTGAT	*	AGTTTATTGAAAGTTAAG
MASS41	GAGAATGAACTTTGAGT	*	AAAATTCAATAGTAAGAGT
HOLL52	GGAGAGAATGAGTTGTAA	*	GTATCAATAAGAATCAAGG

GTAAGAGT is in the 3′ flanking sequence (Table 3). The function of these mirrored sequences is unknown; however, they may be important in preventing folding of the RNA in this region, as mirrored sequences should prevent complementary base pairing.
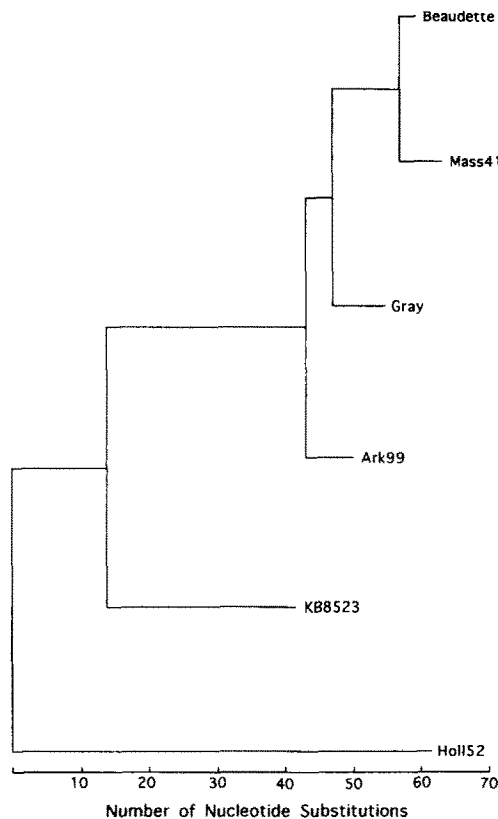

Fig. 4. Phylogenetic tree of 6 strains of IBV based on the sequences of their 3′ non-coding regions.

The sequence relationships of the 3' non-coding regions were examined with a phylogenetic tree (Fig. 4). The Holl52 and KB8523 strains were shown to be more distantly related to the other strains. The 3' non-coding sequences of Mass41, in spite of the absence of the HVR, appeared to be closely related to the Beaudette, and to a lesser extent to the Gray and Ark99 strains. The program used to determine this tree counts the absence of this whole HVR in Mass41 as the result of one recombination event, so only the remainder of the 3' non-coding region of the Mass41 strain was considered for the construction of the phylogenetic tree.

The location of the 3' non-coding HVR is conserved at a position immediately downstream from the stop codon of the nucleocapsid gene. There is no evidence as yet to show that this HVR has any effect on virulence or pathogenesis. There are no detectable protein products from the 3' HVR and no methionines with a preceding putative consensus ribosomal binding site. Since it is completely absent in the Mass41 strain, it would appear that it is not an absolute requirement for viral replication, at least for Mass41. Any specific function of this HVR apparently does not dictate that the sequences be highly conserved but its function may depend on the secondary structure of the RNA. The variability of this HVR as compared to the conservation of the remaining 3' non-coding sequences suggests that the primary sequence itself may not be as functionally important as its influence on the secondary structure of the 3' end of the genome. It is possible that the presence of the HVR may increase the complexity of the secondary structure of the RNA which could in turn affect its association with replication machinery, such as RNA binding proteins.

Acknowledgements

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