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## Sequence analysis of gene 3, gene 4 and gene 5 of avian infectious bronchitis virus strain CU-T2

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### Abstract

We have previously reported the nucleotide sequences of gene 2 (spike (S) protein gene), gene 6 (nucleocapsid (N) protein gene), and the 3' end untranslated region of a novel avian infectious bronchitis virus (IBV) strain, CU-T2 [Jia et al. (1995) Arch. Virol. 140, 259–271]. In the present report we describe the sequences of the remaining genes of this strain (gene 3, 4 and 5) with the exception of gene 1 (RNA polymerase gene). Gene 3 contained three open reading frames (ORFs), 3a, 3b and 3c of 174, 195 and 282 nucleotides (nt), respectively. Gene 4 (membrane (M) protein gene) consisted of 749 nt with a single ORF of 687 nt. Gene 5 contained two ORFs, 5a and 5b, with 198 and 249 nt, respectively. Thus, in total, there were 7349 nt from the 5' end of S protein gene to the 3' end of the CU-T2 genome. The overall nt sequence homologies between gene 3, 4, and 5 of CU-T2 and those of other strains were between 84.1–90.8%, 85.8–88.8% and 90.4–96.4%, respectively. The predicted amino acid (aa) sequence homologies revealed that gene 3b and 5b were more conserved than 3a, 3c and 5a. Each individual gene of CU-T2 strain (with the exception of the RNA polymerase gene) had a different level of homology with the homologous gene of other strains, suggesting that the evolution of IBV strains in general has been a complex, and as yet, poorly understood process. © 1997 Elsevier Science B.V.

**Keywords:** Coronaviridae; Coronavirus; IBV; Virus evolution; Cloning; Recombinant DNA; Membrane protein gene; Small membrane protein gene

### 1. Introduction

Infectious bronchitis virus (IBV), a member of *Coronaviridae* family, has a single-stranded positive-sense RNA genome, which is 27 kb in length (Bournsnell et al., 1987). Gene 1 (RNA polymerase gene) is located on the 5' end of the genome and is about 20 kb long. The remaining 7 kb of the genome consists of five genes: gene 2 (spike glycoprotein (S) protein gene), gene 3, gene 4 [membrane (M) protein gene], gene 5 and gene 6 (nucleocapsid (N) protein gene). In addition, an untranslated region (*UTR*) exists on the extreme 3' end of the genome. Gene 3 and gene 5 contain three (3a,

3b, 3c) and two (5a, 5b) open reading frames (ORFs), respectively. With the exception of gene 3c, which was recently shown to encode small membrane (sM) protein (Liu and Inglis, 1991; Smith et al., 1990), the products of all the other ORFs remain unknown.

Although IBV is the lone virus in the antigenic group III of *Coronaviridae* (Holmes, 1990), more than twenty serotypes within IBV have been recognized worldwide on the basis of virus-neutralization (VN) test (Gelb et al., 1991). IBV strains may undergo substitution of large genomic fragments in multiple genes (Kusters et al., 1990; Wang et al., 1993; Jia et al., 1995). Although the individual genes (mostly S protein genes) of many IBV isolates have been sequenced (Binns et al., 1985, 1986a,b; Bournsnell et al., 1985; Cavanagh and Davis, 1988, 1992; Cavanagh et al., 1992a; Jia et al., 1995; Wang et al., 1993; Williams et al., 1992, 1993), the complete sequence of the 3' end of the genome (whole genome except the RNA polymerase gene) of only one strain (Beaudette strain) of serotype Massachusetts (Mass), and a Japanese strain KB8523 have been deter-

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Abbreviations: aa, amino acid(s); Ark, Arkansas; bp, base pair(s); *Escherichia coli* (*E. coli*); IBV, infectious bronchitis virus; kb, kilobase(s) or 1000 bp; M, membrane; Mass, Massachusetts; N, nucleocapsid; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; ORF, open reading frame(s); S, spike; sM, small membrane; *UTR*, untranslated region.

mined so far (Boursnell et al., 1987; Sutou et al., 1988). The IBV strain CU-T2 described in this report was a unique strain which had apparently originated from Arkansas (Ark) serotype, had undergone recombination in two genes, and expressed both Ark and Mass epitopes on the S1 protein (Jia et al., 1995). In the previous study, we had reported the sequences of the S protein gene, the N protein gene and the 3'-UTR of this strain. Here, we report the sequences of all the remaining 3' end genes of CU-T2: gene 3, M protein gene and gene 5.

## 2. Experimental and discussion

### 2.1. Clones containing cDNA of CU-T2 genes

Cloning of gene 3, M protein gene and gene 5 of CU-T2 are described in Fig. 1. Three overlapping phagemids, pBT2-t223, pBT2-t231 and pBT2-t237, were used for sequencing in this study. pBT2-t223 contained a 2172 bp fragment which overlapped the 3' end of the S protein gene and most of the 5' end of gene 3 (Fig. 1). pBT2-t231 had a 2100 bp insert which overlapped partially pBT2-t223, and contained the entire gene 3, M

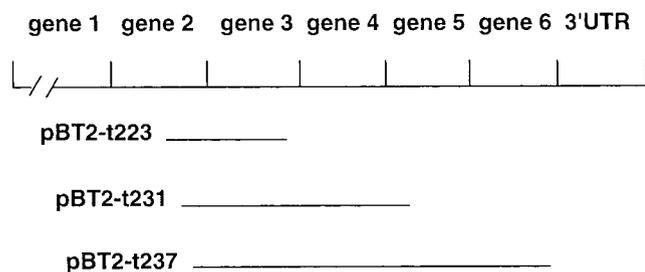


Fig. 1. cDNA clones of CU-T2. IBV strain CU-T2 (Jia et al., 1995) was propagated in 11-day-old specific-pathogen-free (SPF) embryonated chicken eggs. Allantoic fluid collected 40 h after virus inoculation was stored at  $-70^{\circ}\text{C}$ . Virus was purified by sucrose density gradient centrifugation (Sutou et al., 1988), and genomic RNA extracted with phenol-chloroform was used for cDNA synthesis. An oligonucleotide primer complementary to a conserved region of the S genes of several Mass serotype strains containing an *Xho*I restriction site and an eight nucleotide spacer (5'-GAACTAGTCTCGAGGAAGGACGTGGG-ACCTTG-3') was synthesized. This primer was found to bind to downstream 3' end sequences of several genes (i.e., gene 3, M protein gene, and N protein gene) on the IBV genome, therefore it was used for the first strand cDNA synthesis. After blunt-ending the cDNA with T4 DNA polymerase, *Eco*RI adapters were added to the ends of the cDNAs. Following *Eco*RI and *Xho*I digestion, the cDNA fragments were cloned into *Eco*RI/*Xho*I sites in  $\lambda$ -ZAP vector (Stratagene, La Jolla, CA, USA). The cDNA library was packaged using Gigapack II Gold packaging extract (Stratagene). *Escherichia coli* (*E. coli*) containing the library were plated onto NZY plates and duplicated onto nitrocellulose filters. The positive plaques were identified by hybridization with a 5' end-radiolabeled probe prepared from a *Sty*I fragment derived from plasmid pBSM41M, which contained the S gene of Mass41. Phagemids containing CU-T2 genes were obtained by *in vivo* excision in (*E. coli*) strain XL-1 using a protocol furnished by Stratagene. A total of 27 positive clones were obtained. Three of them were characterized.

protein gene, and partial 5' end of gene 5 (Fig. 1). pBT2-t237 contained a 3550 bp fragment which overlapped a region extending from the 3' end of S protein gene to the 3' end of N protein gene (Fig. 1).

### 2.2. Sequences of gene 3, M protein gene, gene 5

Gene 3 of CU-T2 consisted of 711 nt, which contained three ORFs, 3a, 3b and 3c. The ORFs consisted of 174, 195 and 282 nt (potentially encode 58, 65 and 94 aa), respectively. 32 nt of the coding sequence of the S protein gene overlapped gene 3. M protein gene consisted of 749 nt, and with a single ORF of 687 nt (encoded 229 aa). There were 66 nt overlapping between the 3' end of ORF 3c and 5' end of M protein gene. There was a non-coding region consisting of 342 nt between 3' end of M protein gene and gene 5. Gene 5 had 460 nt, and contained two ORFs (5a and 5b) which consisted of 198 and 249 nt (potentially encoded 66 and 83 aa), respectively. 159 nt of ORF 5b overlapped the 5' end sequence of N protein gene (see Fig. 2).

### 2.3. Comparison of the sequences

Six IBV strains whose nt sequences of the gene 3 are available, are listed in Table 1. Of those strains, UK/183/66 and UK/68/84 are non-Mass serotype strains (Cavanagh et al., 1992b), whereas Portugal/322/82 strain belongs to Mass serotype (Cavanagh et al., 1992b). However, the latter strain and strain KB8523 were possible recombinants, with recombinant fragments located between 3' end of S protein gene and gene 3. (Cavanagh et al., 1992b; Sutou et al., 1988). The overall nt sequence of CU-T2 gene 3 compared with sequences of the six strains listed in Table 1, which showed homologies between 84.1% and 90.8%. Except Portugal/322/82 and KB8523, however, there were only about 85% homologies between the CU-T2 and the prototype Mass strains (i.e., Mass41 and Beaudette) or the non-Mass and non-Ark serotype. It was earlier reported that some non-Mass IBV isolates contained deletions in the 3' end of gene 3, and, as an exception, the Mass serotype Portugal/322/82 also had a similar deletion (Cavanagh and Davis, 1988; Cavanagh et al., 1992b). In this study, CU-T2 was found to have a 23 nt deletion in that region compared to a 32 nt deletion in Portugal/322/82, a 9 nt deletion in UK/68/84, and a 6 nt deletion in UK/183/66. Thus, results of the percentage homology and the deletions in gene 3 nt sequences suggested that Ark-like IBV has a relatively distant evolutionary relationship with the Mass serotype strains and non-Mass and non-Ark serotype. Besides the nt sequences, the deduced aa sequences of gene 3a, 3b and 3c of CU-T2 were also compared with published sequences (Table 1). The overall aa sequence homologies of 3a (between 81% and 86.2%) and 3c (between 84% and 90.4%) were close to

<sup>gene 3</sup>  
 1 ACTGAAACAAT ACAGACCTAA AAGTCTGTT TAATGATTCA CACCCCAATA  
 50 TCITTTCTTAA TAGTGTTAAC TCTTCTTTGG TGTAACCTTG TATTAAGTTG  
 100 TTTTAAAGAG TGTGTTT TAG CGCTTTTGCA AGTAATACAA GTTTTACTCC  
 150 AAATCATTAG TAATAACTTA CAGTCTAGAC TTCTGCTTTG GCACAGTCTA  
<sup>3b</sup>  
 200 GACTAATGTT AGATTTTGAA GCAATTATTG AAAGTGTCA GCAATAAAT  
 250 CAGCAATCA GTTTTCGATT ACAGCACATT TCAAGTGTGC TAAGCACTGA  
 300 ATTATTGAC CCCTTTGAAG TCTGTGTTA CAGAGGAGGT AATTATTGGG  
<sup>3c</sup>  
 350 AGTTAGAGTC AGCTGACGAG TTTTCAGGTG ATGACGAATT TATTGAGTAA  
 400 ATCGCTAGAG GAGAACGGAA GTTTCCTAAC ACGAGTTTAC ATATTGTTG  
 450 GATTGTAGC ACITTACCTA CTAGGTAGAG CACTCCAAGC ATTTGTAACA  
 500 GCTGCCGACG CTGTGCGTTT ATTTGGTAT ACATGGGTAA TAGTTCCTGG  
<sup>gene 4</sup>  
 550 AGCCAAGGGT ACAGCCTTTG TATAAATTA TACATATGGT AAACAACTTA  
 600 ACAACC<sup>3</sup>GGGA TTTAGAAACG GTTATTGTTA ACGAGTTTCC CAAAAACGGT  
 650 TGGAAACAATGAATGATA ATGAATTGCA CACTTAAATC TCAGCAGCAA  
 700 GCAGCTGAGC TTTTAAAGA ATACAATGTT TTTGTAACGG CTTTCCTTTT  
 750 GTTCTTAGT AIACTACTTC AGTAGGGTA CGCAACIAGA AGTCGGTTTA  
 800 TTTATATACT GAAAATGATA TTGTATGGT GTTTTGGCC CCTTAACAAT  
 850 GCAGTAGGTG TAATTTTCATG TATATCCCA CCAAACACAG GAGGGTCTTT  
 900 TGTCGCAGCG ATAATACTTA CAGTGTGTGG GTGCTTTGTC TTTTCTTGG  
 950 GTTATTGGAT CCAGAGTATC AGACTCTTTA AGCGGGGTAG GTCATGGGGG  
 1000 GCCATTAAC CCGGATCTTA TGCCGGAGGT CCAATACTCC TAACATAATGG  
 1050 CCAACAATGT AATTTTGCTA TAGAGAGTGT CCCAGTGGTG CTTTCCCAA  
  
 1100 TTATAAAGAA TGGTGTCTTT TATTGTGAGG GCCAGTGGCT TGCTAAGTGT  
 1150 GAACCAGACC ACTTGCCTAA AGATATATTT GTTTGTACAC CGGATAGACG  
 1200 TAATATCTAC CGTATGGTGC AGAAATATAC TGGTGACCAA AGCGGAAATA  
 1250 AGAAACGTTT TGCTACGTTT GTCTATGCAA AGCAGTCAGT AGATACTGGC  
 1300 GAGCTAGAAA GTGTAGCAAC AGGAGGGAGT AGTCTTTACA CATAAATGTTG  
 1350 TGTGTGTAGA GAGTATTAA AATTATCTT TAATAGTGCC TCTAFTTAA  
 1400 GAGCGCAIAA TAGTATTATT TTTGAGGATA TTAATATAAA TCCTCTGTT  
 1450 TTACTACTCT TTTCAAGAG CTATTATTTA AAAAAACAGTT TTTCCACTCT  
 1500 TTTGTGCCAA AAACCTGTGT TGTTAATGGT GTAACCTTTC AAGTAGATAA  
 1550 TGGA<sup>5</sup>AAGTC TACTTACGAA GGAACACCAG TTTTCCAAAA AGGTGTGTTG  
 1600 AGAATGTGGT CCAATTATAA GAAAGATTAG AATAATTAAG TCACCTACTA  
 1650 CACTTATTTT TATAAGAGGC GTTTTATTTT ACAAACCTTAACAATACG  
<sup>5a</sup>  
 1700 GACGATGAAA TGGCTGACTA TTTTGGAAAG AGCAGTATT TCAATGTATA  
 1750 AAGCCCTACT ATTAACCTCAA TTAAGAGTGT TAGATAGGTT AATTCTAGAT  
 1800 CACGGACCAA AACCGGTTTT AACGTGTAGT AGCGGAGTGC TTTTGTTCAC  
<sup>5b</sup>  
 1850 GTTAGATTTA GTTTCTCGGT TGGCGTTTAC GCCACCCAA TCCTGGTAT  
 1900 GAAATAAGT AAAGATAATC CTTTTCGCGG AGCAATAGCA AGAAAAGCGC  
<sup>gene 6</sup>  
 1950 GAATTTATCT TAGAGAAGGA TTAGATTGTG TTTACTTT CTTAACAAGCA  
 2000 GGACAAGCAG AGCCTTGTCC CGCGGTGACC TCTCTAGTAT TCCAAGGGAA  
 2050 AACTTGTGAG GAACACATAA ATAATAATAA TCTTTTGTCA TGCCAAGCGG  
 2100 TAAGGCAACT GGAAAGACAG ACGCCCCAGC GCCAGTCATC AAACTAG

those of the respective nt sequences. However, the homologies of putative 3b aa sequences were between 87.5% and 95.4%, which showed an average of 6.5% and 4.1% higher homologies than 3a and 3c, respectively. The result suggested that the 3b region is relatively more conserved compared to regions 3a and 3c. In addition, a hydrophobic region found at the amino-terminal region of the 3c protein, which was adjacent to a cysteine-rich region in CU-T2, was similar to that found in other coronaviruses (Siddell, 1995).

Although the M protein gene of IBV was believed to be a conserved gene, comparison of nt sequences of CU-T2 M protein gene to those of three available IBV strains showed only 85.8%, 86.6% and 88.8% homology, respectively (Table 1). The aa sequences, however, showed higher homologies (90.3%, 92% and 90.7%, respectively). Three insertions (each encoding an additional aa) were found in the first 240 nt of M protein gene of CU-T2 compared to both Beaudette and KB8523 strains.

Overall nt sequence of gene 5 of CU-T2 showed 90.4% and 96.4% homology to those of Beaudette and KB8523 (Table 1). The aa sequence homology between gene 5a of CU-T2 and that of the other two strains was between 87.9% and 92.4%, and that of gene 5b was 95.2% and 97.6%, respectively (Table 1). The sequence of 5b showed 7.3% and 5.2% higher homologies than those of 5a, indicating that the 5b region was probably more conserved than the 5a region.

The sequences of gene 3, M protein gene and gene 5 of CU-T2, and those of S protein gene, N protein gene and the 3'-UTR reported previously by us (Jia et al., 1995), provided all the sequences of the genome except the RNA polymerase gene. A total of 7349 nt were found in a region beginning from the 5' end of S protein gene to the 3' end of CU-T2 genome, compared to 7355 and 7301 nt reported for KB8523 and Beaudette strains, respectively. The sequence CTGAACAA found at the starting sites of S protein gene and gene 3 of CU-T2, and the sequence CTTAACAA found at the start of M protein gene, gene 5 and N protein gene, respectively, were identical to those found in the identical sites in

Fig. 2. Nucleotide sequences of gene 3, M protein gene, and gene 5 of IBV CU-T2. Heavy underlines indicate the putative start codons. Light lines above the sequence indicate the stop codons. The conserved nt sequence CTGAACAA or CTTAACAA, which is located at the starting site of each gene, is boxed. Three overlapping phagemids pBT2-t223, pBT2-t231 and pBT2-t237 containing CU-T2 genes were used for DNA sequencing which was carried out by the dideoxy method using a Sequenase 2.0 DNA sequencing kit (US Biochemical, Cleveland, OH, USA). The nucleotide sequences reported here have been deposited with the GenBank. The accession Nos. are as follows: Gene 3 of IBV CU-T2, U46036; M protein gene (gene 4) of IBV CU-T2, U46035; Gene 5 of IBV CU-T2, U46037; Complete sequences of the 3' end genome (whole genome except the RNA polymerase gene) of IBV CU-T2, U49858.

Table 1

Percentage nt and aa homologies between coding regions of gene 3, M protein gene, and gene 5 of CU-T2 and other IBV strains

IBV strains	Percent homology with CU-T2								
	Gene 3				M protein gene		Gene 5		
	nt (%)	aa (%)			nt (%)	aa (%)	nt (%)	aa (%)	
		3a	3b	3c				5a	5b
Portugal/322/82 <sup>a</sup>	90.8	86.2	95.4	88.3	N/A	N/A	N/A	N/A	N/A
KB8523 <sup>b</sup>	90.2	84.5	93.9	90.4	85.8	90.3	96.4	92.4	97.6
UK/183/66 <sup>a</sup>	86.7	87.9	92.2	86.2	N/A	N/A	N/A	N/A	N/A
Mass41 <sup>c</sup>	84.8	81.0	90.8	88.3	N/A	N/A	N/A	N/A	N/A
Beaudette <sup>d</sup>	84.6	84.5	89.2	87.2	88.8	90.7	90.4	87.9	95.2
UK/68/84 <sup>a</sup>	84.1	86.2	87.5	84.0	N/A	N/A	N/A	N/A	N/A
68/2 <sup>e</sup>	N/A	N/A	N/A	N/A	86.6	92.0	N/A	N/A	N/A

<sup>a</sup>Cavanagh et al. (1992a,b); <sup>b</sup>Sutou et al. (1988); <sup>c</sup>Niesters et al. (1986); <sup>d</sup>Bournnell et al. (1987); <sup>e</sup>Binns et al. (1986b). N/A, not available.

Beaudette and KB8523 strains. The overall sequence of the CU-T2 genome distal to the RNA polymerase gene was found to have 87% homology with the equivalent regions of the Beaudette and KB8523 strains (compared with 92% homology between the latter two strains themselves). This suggested once again that Ark-like IBV probably has a relatively distant evolutionary relationship with the Mass as well as non-Mass and non-Ark serotypes. Table 1 showed that the nt sequence homologies between gene 3, M protein gene and gene 5 of CU-T2 and Beaudette strain were 84.6%, 88.8% and 90.4%, respectively, while the homologies between CU-T2 and KB8523 were 90.2%, 85.8% and 96.4%. Furthermore, nt and aa sequences of other individual genes of CU-T2, Beaudette and KB8523 showed different degrees of homology (data not shown), implying that the evolution of the IBV strains might have involved a complex series of events, which are as yet poorly understood.

### 3. Conclusions

- (1) The gene 3, M protein gene and gene 5 of IBV strain CU-T2 were cloned and sequenced. Gene 3 had 711 nt and three potential ORFs, 3a, 3b and 3c. The ORFs consisted of 174, 195 and 282 nt, respectively. M protein gene had 749 nt, and a single ORF of 687 nt. Gene 5 had 460 nt and two ORFs, 5a and 5b, of 198 and 249 nt, respectively. There was a non-coding region between M protein gene and gene 5 consisting of 342 nt. In total, there were 7349 nt from the 5' end of S protein gene to the 3' end of the CU-T2 genome.
- (2) The nt and aa sequences of CU-T2 were compared with published sequences of seven other IBV strains. The overall nt sequence homologies between gene 3, M protein gene and gene 5 of CU-T2 and other

strains were between 84.1% and 90.8%, 85.8% and 88.8%, 90.4% and 96.4%, respectively. The sequence of the CU-T2 genome distal to the RNA polymerase gene was found to have only an 87% homology with those of the Beaudette and KB8523 strains. The deletion observed in gene 3 nt sequences of CU-T2 suggested that Ark-like IBV has a relatively distant evolutionary relationship with the Mass and non-Mass and non-Ark serotype. In addition, the results of aa sequence comparisons suggested that the gene 3b and 5b were relatively more conserved than 3a, 3c and 5a.

- (3) The nt sequence homologies between gene 3, M protein gene and gene 5 of CU-T2 and Beaudette strain were 84.6%, 88.8% and 90.4%, respectively, while homologies between the same three genes of CU-T2 and KB8523 were 90.2%, 85.8% and 96.4%. Furthermore, nt and aa sequences of other individual genes of CU-T2, Beaudette and KB8523 showed different degrees of homology, implying that the evolution of the IBV strains might have involved a complex series of events.

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