Genomic Characteristics and Changes of Avian Infectious Bronchitis Virus Strain CK/CH/LDL/97I after Serial Passages in Chicken Embryos

Fei Zhao  Zongxi Han  Tingting Zhang  Yuhao Shao  Xiangang Kong  
Huijie Ma  Shengwang Liu

Division of Avian Infectious Diseases, State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, The Chinese Academy of Agricultural Sciences, Harbin, PR China

Key Words
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Abstract
Background: We previously attenuated the infectious bronchitis virus (IBV) strain CK/CH/LDL/97I and found that it can convey protection against the homologous pathogenic virus.

Objective: To compare the full-length genome sequences of the Chinese IBV strain CK/CH/LDL/97I and its embryo-passage, attenuated level to identify sequence substitutions responsible for the attenuation and define markers of attenuation.

Methods: The full-length genomes of CK/CH/LDL/97I P5 and P115 were amplified and sequenced. The sequences were assembled and compared using the MEGALIGN program (DNAStar) and a phylogenetic tree was constructed using MEGA4 software.

Results: The CK/CH/LDL/97I virus population contained subpopulations with a mixture of genetic mutants. Changes were observed in nsp4, nsp9, nsp11/12, nsp14, nsp15, nsp16, and ORF3a, but these did not result in amino acid substitutions or did not show functional variations. Amino acid substitutions occurred in the remaining genes between P5 and P115; most were found in the S region, and some of the nucleotide mutations resulted in amino acid substitutions. Among the 9 nsps in the ORF1 region, nsp3 contained the most nucleotide substitutions.

Conclusions: Sequence variations in different genes, especially the S gene and nsp3, in the genomes of CK/CH/LDL/97I viruses might contribute to differences in viral replication, pathogenicity, antigenicity, immunogenicity, and tissue tropism.

Introduction
Coronaviruses (CoVs) infect a variety of animals and can cause respiratory, enteric, hepatic and neurological diseases of varying severities. CoVs were recently divided into four genera based on genotypic and serological characterizations: alpha, beta, gamma, and delta. Infectious bronchitis virus (IBV) is a Gammacoronavirus of the subfamily Coronavirusae, family Coronaviridae, order Nidovirales [1]. IBV is distributed worldwide, it is highly infectious, and extremely difficult to control because of its extensive genetic diversity, short generation time, and high mutation rate. Although live-attenuated infectious bronchitis (IB) vaccines have been used worldwide since the 1950s, IBV continues to cause disease in chickens, even in vaccinated birds, because the virus is constantly changing.
and evolving to avoid the host immune response. IBV can cause respiratory disease in chickens of all ages [2]. Some strains are nephropathogenic, resulting in renal-induced mortality of 25–80% in susceptible flocks [3]. Other strains can replicate in the oviduct and cause permanent damage in immature females or pullets resulting in limited egg production in mature hens [2].

CoVs are enveloped viruses that replicate in the cell cytoplasm and contain an unsegmented, single-stranded, positive-sense RNA genome of 28–32 kb, with a 5′ cap and a 3′ polyA tail [4]. Like all CoVs, the proximal two thirds of the IBV genome encode two overlapping open reading frames (ORFs), 1a and 1b, which are proteolytically cleaved by two virus-encoded replicase proteins, the papain-like and 3C-like proteinases, into at least 15 non-structural proteins (nsp2–16) [4]. Replication of the CoV RNA genome and transcription of a nested set of 5–8 subgenomic RNA species are carried out by these proteinases. However, the exact functions of individual replicase proteins remain largely unknown. The remaining third of the IBV genome encodes four major structural proteins: the spike (S) glycoprotein, the small envelope (E) protein, the membrane (M) glycoprotein, and the nucleocapsid (N) protein. Two small accessory genes, genes 3 and 5, have been described in IBV, which express accessory proteins 3a, 3b and 5a, 5b, respectively [3, 5, 6]. In addition, the 5′ and 3′ untranslated regions (UTRs) in the IBV genome, like those of other CoVs, usually harbor important structural elements involved in replication and/or translation [7–11].

IB is mainly controlled by the use of live-attenuated vaccines derived from virulent viruses prevalent in a particular country or region by multiple serial passages in 9- to 11-day-old embryonated chicken eggs [12–16]. As a consequence of this process, the virus becomes more adapted and more highly pathogenic to the embryo, with concomitant attenuation in mature chickens. The IBV strain Beaudette was attenuated after several hundred passages in embryonated hen’s eggs [17] resulting in loss of virulence and also potential loss of immunogenicity. A similar result was observed in IBV strain CK/CH/LHL/04V, the immunogenicity of which was substantially decreased after 110 passages in embryonated hen’s eggs, resulting in loss of virulence to chicks [16]. However, most attenuated IB vaccine strains, such as H120, maintain their immunogenicity after adaptation to embryonated chicken eggs [12–15], though the genomic mutations in passaged strains associated with attenuation of pathogenicity, immunogenicity, tissue tropism, and replication capacity in chicken tissues are unknown and variable, leading to differing efficacies associated with different vaccines.

A novel type of IBV, designated as CK/CH/LDL/97I, emerged in the mid-1990s and circulated among both vaccinated and non-vaccinated chicken flocks in China, causing 100% morbidity and about 10% mortality in young infected chicks. In recent years, this type of IBV has also been found in Taiwan [18] and regions of the Middle East, resulting in severe losses to both the layer and broiler industries [19]. Both experimental and field studies suggested that vaccination using available commercial vaccines provided poor protection against this type of virus, emphasizing the need for the development of an efficacious, live-attenuated vaccine against CK/CH/LDL/97I to prevent and control this type of IBV [20]. We previously attempted to develop an IB vaccine by serial passage of the IBV strain CK/CH/LDL/97I in embryonated eggs [21]. Although the vaccination challenge test showed that the attenuated P115 virus could provide complete protection against the homologous pathogenic parent virus P5, in terms of clinical response and virus recovery in the trachea and kidney [20, 21], the genomic changes in the pathogenic parental CK/CH/LDL/97I strain and its attenuated virus, and the genomic changes associated with attenuation of pathogenicity, immunogenicity, tissue tropism, and replication capacity after serial passages remain unknown.

Although it is possible to study the evolution of viruses and the subsequent impact on viral characteristics by comparing genomic sequences between heterologous strains, analysis of homologous strains provides a unique opportunity to elucidate the specific genes or gene sites involved in viral characteristics, such as pathogenicity, immunogenicity, and tissue tropism. To identify specific sequence changes responsible for adaptation of the field IBV isolate to chicken embryonic tissue and subsequent attenuation, the whole viral genome of the IBV P5 pathogenic parental CK/CH/LDL/97I strain was sequenced and compared with the attenuated, P115 level virus, to provide a better understanding of the relationship between the genomic differences and related characteristics of the pathogenic and attenuated viruses.

Materials and Methods

Eggs and Viruses

Fertile White Leghorn specific pathogen-free (SPF) chicken eggs were obtained from the Laboratory Animal Center, Harbin Veterinary Research Institute, the Chinese Academy of Agricultural Sciences, Harbin, China.
The pathogenic IBV strain CK/CH/LDL/971 (P5), previously characterized as belonging to the CK/CH/LDL/971 type [21], and its embryo-passaged, attenuated virus (P115) [20] were used in this study. CK/CH/LDL/971 was isolated in 1997 from the swollen proventriculi of broiler chickens and propagated following standard procedures at 37°C in the chorioallantoic cavities of 9- to 11-day-old SPF embryonated eggs. This attenuated virus can provide complete protection against challenge of the parental CK/CH/LDL/971 strain according to clinical response and virus recovery in tracheas and kidneys [20, 21].

The virus stocks of CK/CH/LDL/971 P5 and P115 were produced by inoculating embryonated SPF chicken eggs via the allantoic cavity with the virus, and by collecting the infectious allantoic fluid 72 h after inoculation, respectively, as described previously [21]. Each virus was identified by electron microscopy, reverse transcriptase-polymerase chain reaction (RT-PCR), and sequencing of the entire S1 protein gene, as described previously [21], before aliquoting and storage as virus stocks at –80°C. The infectious virus titer was determined by inoculation of SPF embryonated eggs following the Reed and Muench method in MEGA4 software (www.megasoftware.net) at a bootstrap value of 1,000 replicates. The selected avian CoV reference strains and their accession numbers are shown in online supplementary table 2.

In addition, another 15 overlapping pairs of primers covering the entire S1 protein gene, as described previously [21], before aliquoting and storage as virus stocks at –80°C. The infectious virus titer was determined by inoculation of SPF embryonated eggs following the Reed and Muench method in MEGA4 software (www.megasoftware.net) at a bootstrap value of 1,000 replicates. The selected avian CoV reference strains and their accession numbers are shown in online supplementary table 2.

Cloning and Sequencing of CK/CH/LDL/971 P5 and P115 Genomes

Viral RNA was extracted from 200 µl of CK/CH/LDL/971 P5 and P115 virus stocks, respectively, using TRIzol reagent (Invitrogen, Grand Island, N.Y., USA) according to the manufacturer’s instructions. RNA was air-dried for 2–10 min, re-dissolved in 30 µl of RNase-free water, and stored at –70°C for further use. The initial results (data not shown) revealed that primers N (–) contained sequences consistent with those of CK/CH/LDL/971, and reverse transcription (RT) was therefore performed with M-MLV reverse transcriptase (Invitrogen) using the primer N (–). RT procedures were performed using 40 µl of RNA in an 80-µl reaction volume, as previously described [16]. Each cDNA fragment was PCR-amplified from the RT products, as described previously [16]. PCR products were purified from agarose gels using a DNA extraction kit (Boehringer Mannheim GmbH, Mannheim, Germany) and cloned into the pMD18-T vector (TaKaRa Bio, Inc., Dalian, China), following the manufacturer’s instructions.

Fourteen pairs of overlapping primers were used for PCR amplification. The sequences and locations of the primers used in this study are listed in online supplementary table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000365193). RNA extraction, cDNA generation, PCR amplification, and gene-fragment cloning and sequencing were conducted independently for each of the two passages [16]. The 3′ and 5′ ends of the viral genomes were confirmed by rapid amplification of cDNA ends (RACE) using a 3′/5′ RACE kit (TaKaRa Bio, Inc.), according to the manufacturer’s instructions. PCR products were purified from agarose gels using a DNA extraction kit (Boehringer Mannheim GmbH) and cloned into the pMD18-T vector (TaKaRa Bio, Inc.), following the manufacturer’s instructions. A total of five clones of each gene fragment were selected and sequenced for each of the passages to exclude errors due to RT and PCR reactions. Sequences were assembled using the MEGALIGN program (DNASTar, Madison, Wis., USA) and edited manually to produce final sequences of the viral genomes.

In addition, another 15 overlapping pairs of primers covering the full-length genome of IBV were designed (online suppl. ta-
| IBV gene | Genome position<sup>a</sup> | Position in gene<sup>b</sup> | Nucleotide change | Codon change | Amino acid substitution |
|----------|-----------------------------|-----------------------------|------------------|--------------|------------------------|
| 5′-UTR   | 53                          | –                           | T→C              | –            | –                      |
|          | 93                          | –                           | C→T              | –            | –                      |
| ORF1 nsp1/2 | 837                        | 309                         | T→C              | GTT→GTC      | Val→Val               |
|          | 1160                        | 632                         | C→T              | GCA→GTA      | Ala→Val               |
|          | 1700                        | 1172                        | T→C              | GTT→GCT      | Val→Ala               |
|          | 2263                        | 1735                        | C→T              | CTT→TTC      | Leu→Phe               |
| nsp3     | 2685                        | 138                         | A→A(7)/G(3)      | GAA→GAA(7)/GAG(3) | Glu→Glu                |
|          | 5073                        | 2526                        | T→T(6)/G(4)      | CAT→CAT(6)/CAG(4) | His→His(6)/Gln(4)     |
|          | 5261                        | 2714                        | G→A              | GGA→GAA      | Gly→Glu               |
|          | 5331                        | 2787                        | C→T(6)/C(4)      | GAC→GAT(6)/GAC(4) | Asp→Asp               |
|          | 6480                        | 3936                        | T→T(6)/C(3)      | CCT→CCT(7)/CCC(3) | Pro→Pro                |
|          | 6503                        | 3959                        | A→A(7)/G(3)      | GAA→GAA(7)/GGA(3) | Glu→Gly               |
|          | 7204                        | 4660                        | T→T(7)/C(3)      | TGT→TGT(7)/CGT(3) | Cys→Cys(7)/Arg(3)      |
| nsp4     | 8331                        | 1005                        | G(7)/T(3)→T     | TGG(7)/TGT(3)/TGT | Try(7)/Cys(3)→Cys(3)  |
| nsp5     | –                           | –                           | –                | –            | –                      |
| nsp6     | –                           | –                           | –                | –            | –                      |
| nsp7     | –                           | –                           | –                | –            | –                      |
| nsp8     | –                           | –                           | –                | –            | –                      |
| nsp9     | 11684                       | 137                         | C(7)/T(3)→C     | TCA(7)/TTA(3)→TCA | Ser(7)/Leu(3)→Leu     |
| nsp10    | –                           | –                           | –                | –            | –                      |
| nsp11/12 | 12669                       | 354                         | G(7)/A(3)→G(7)/A(3) | ATG(7)/ATA(3)→ATG(7)/ATA(3) | Met(7)/Ile(3)→Met(7)/Ile(3) |
|          | 13143                       | 828                         | C→C(7)/T(3)     | CTC→CTC(7)/CTT(3) | Leu→Leu               |
|          | 13201                       | 886                         | T(7)/C(3)→T     | TTA(7)/CTA(3)→TTA | Leu→Leu               |
|          | 13312                       | 997                         | C(7)/T(3)→C     | CTT(7)/TTT(3)→CTT | Leu(7)/Phe(3)→Leu     |
|          | 13581                       | 1266                        | C→A(7)/A(3)     | GTC→GTA(7)/GTC(3) | Val→Val               |
|          | 13875                       | 1560                        | T→G(7)/T(3)     | AAT→AAG(7)/AAT(3) | Asn→Lys(7)/Asn(3)     |
|          | 14025                       | 1710                        | C(7)/T(3)→T(7)/C(3) | TCC(7)/TCT(3)→TCT(7)/TCC(3) | Ser→Ser               |
| nsp13    | 15713                       | 579                         | G(7)/T(3)→G(3)  | GTG→GTT(7)/CTG(3) | Val→Val               |
|          | 15824                       | 690                         | C(7)/T(3)→C     | CAC(7)/CAT(3)→CAC | His→His               |
|          | 15859                       | 725                         | T→C              | CTA→CCA      | Leu→Pro               |
|          | 16255                       | 1121                        | A(7)/G(3)→A     | GAT(7)/GGT(3)→GAT | Asp(7)/Gly(3)→Asp     |
|          | 16306                       | 1172                        | C(7)/T(3)→C     | ACA(7)/ATA(3)→ACA | Thr(7)/Ile(3)→Thr    |
|          | 16879                       | 1745                        | G(7)/A(3)→A     | AGT(7)/AAT(3)→AAT | Ser(7)/Asn(3)→Asn    |
|          | 16919                       | 1785                        | T(7)/A(3)→T     | AGT(7)/AGA(3)→AGT | Ser(7)/Arg(3)→Ser    |
| nsp14    | 18086                       | 1152                        | G(7)/A(3)→G     | GTG(7)/GTA(3)→GTG | Val→Val               |
|          | 18200                       | 1266                        | C→C(7)/T(3)     | CAC→CAC(7)/CAT(3) | His→His               |
|          | 18318                       | 1384                        | C(7)/T(3)→C     | CTA→TTA(7)/CTA(3) | Leu→Leu               |
| nsp15    | 18713                       | 216                         | G(7)/A(3)→G     | CTG(7)/CTA(3)→CTG | Leu→Leu               |
|          | 18772                       | 275                         | C(7)/T(3)→T     | ACA(7)/ATA(3)→ACA | Thr(7)/Ile(3)→Thr    |
|          | 18881                       | 384                         | A(7)/C(3)→A(5)/C(5) | GCA(7)/GCC(3)→GCA(5)/GCC(5) | Ala→Ala               |
|          | 19294                       | 797                         | A→A(7)/G(3)     | CAA→CAA(7)/CGA | Gln→Gln(7)/Arg(3)     |
|          | 19356                       | 859                         | C→C(7)/T(3)     | CTG→CTG(7)/TTG(3) | Leu→Leu               |
| nsp16    | 20309                       | 798                         | G→A              | AAG→AAA      | Lys→Lys               |
|          | 20351                       | 840                         | C→C(7)/T(3)     | AAC→AAC(7)/AAT(3) | Asn→Asn               |
|          | 20415                       | 904                         | A→G(7)/A(3)     | ATG→GTG(7)/ATG(3) | Met→Val(7)/Met(3)     |

Table 1. Nucleotide and amino acid substitutions identified in IBV CK/CH/LDL/97I P5 compared to the embryo-passaged, attenuated P115 strain.
Table 1 (continued)

| IBV gene | Position in gene | Nucleotide change | Codon change | Amino acid substitution |
|----------|------------------|-------------------|--------------|-------------------------|
| ORF2 S1  |                  |                   |              |                         |
| (S gene) |                  |                   |              |                         |
| ORF2 S2  |                  |                   |              |                         |
| ORF3 3a  |                  |                   |              |                         |
| 3b       |                  |                   |              |                         |
| 3c       |                  |                   |              |                         |
| ORF3 3b  |                  |                   |              |                         |
| ORF3 3c  |                  |                   |              |                         |
| ORF4     |                  |                   |              |                         |
| (M gene) |                  |                   |              |                         |
| ORF5     |                  |                   |              |                         |
| M-ORF5   |                  |                   |              |                         |
| ORF6     |                  |                   |              |                         |
| (N gene) |                  |                   |              |                         |
| 3′-UTR   |                  |                   |              |                         |

*a* Based on the sequence of the IBV CK/CH/LDL/97I P5 genome, GenBank accession No. JX195177.

*b* Position of nucleotides in each gene.

*c* A 2-bp sequence, TA, was inserted in CK/CH/LDL/97I P115, compared to that of the P5 sequence, in the position between the M gene and ORF5. The position of the 2-bp sequence in CK/CH/LDL/97I P115 is shown.
the genetic diversity within the populations of the viral RNAs from a given passage.

The dominant sequences of both CK/CH/LDL/97I P5 and P115 were assembled into one contiguous sequence to represent the entire viral genome, irrespective of the subpopulations. Sequences of 27,680 and 27,682 nucleotides were obtained from CK/CH/LDL/97I P5 and P115, respectively, excluding the polyadenylation tail at the 3′ end. P115 had two nucleotide insertions in the intergenic UTR between M and gene 5, compared to P5 (table 1; fig. 1 a, b). The genomes of both viruses were similar in terms of overall coding capacity and genomic organization to those of other IBVs. The genome organization of CK/CH/LDL/97I was as follows: 5′-UTR-gene 1 (ORF1a, 1b)-S-gene 3 (ORF3a, 3b, E)-M-gene 5 (ORF5a, 5b)-N-UTR-3′.

Substitutions in Genomic Sequence of CK/CH/LDL/97I after Serial Passage

Subpopulations were identified in both CK/CH/LDL/97I P5 and P115, evidenced by the variations in genomic sequences of the viruses (table 1). As summarized in table 1, no sequence substitutions were observed in nsp5, nsp6, nsp7, nsp8, nsp10, ORF3b, or ORF5a after 115 passages. Substitutions were observed in nsp4, nsp9, nsp11/12, nsp14, nsp15, nsp16, and ORF3a, but these did not result in amino acid substitutions and/or variations. Amino acid substitutions occurred in the remaining genes between P5 and P115. Two and three nucleotide substitutions were observed, respectively, in the 5′- and 3′-UTRs of CK/CH/LDL/97I P115, compared to the parental strain P5. Most nucleotide mutations were found in the S region between P5 and P115, and some of the nucleotide mutations resulted in amino acid substitutions. More mutations occurred in the S2 gene than in the

Fig. 1. Multiple nucleotide (a) and amino acid (b) sequence alignments of the intergenic UTRs of IBV CK/CH/LDL/97I P5 and P115. The lengths of the intergenic UTR from P5 and P115 were 361 and 363 bp in length, respectively. The initiation and stop codons are underlined. The two nucleotide insertions in P115 are boxed.
Fig. 2. A phylogenetic tree constructed based on the full-length genomic sequences of IBVs using the neighbor-joining method. IBV CK/CH/LDL/971 P5 and P115 are boxed. Each of the reference strains are indicated and the countries where the IBV strains were isolated are shown in parentheses.
S1 gene. Among the 9 nsps in the ORF1 region, which showed nucleotide alterations between the pathogenic P5 and attenuated P115 viruses, nsp3 had the most.

**Discussion**

In this study, CK/CH/LDL/97I was unexpectedly grouped together with the US IBV strains, but was distinct from all other IBV strains reported so far in China. CK/CH/LDL/97I emerged in the mid-1990s as a novel IBV type in China [21, 23] and was associated with IB outbreaks on the Liaotung Peninsula between 1997 and 2001. This isolate seems to have high affinity for the proventriculus in chickens and is implicated in a novel pathogenicity associated with transmissible proventriculitis [23], though few experimental proventriculitis models have been generated using this type of IBVs. The origin of this virus type remains unknown and no complete genomic sequence for it is available in the GenBank database. The lack of sufficient information limits the comprehensive tracing of the origin and evolution of the CK/CH/LDL/97I strain, but it is noteworthy that this type of IBV was also found in Taiwan [18] and regions of the Middle East in recent years [19]. We identified a close genetic relationship between CK/CH/LDL/97I and the Delaware-type IBVs, which was isolated in the US, by entire genome analysis, suggesting a common origin or epidemiological link between the CK/CH/LDL/97I and Delaware-type IBV strains.

It has been suggested that minor differences near the receptor-binding domain of the S1 subunit of the spike protein could modify the affinity and virulence of CoVs for different cell surface glycoproteins [24–26]. Consequently, the S protein has been shown to be correlated to the virulence attenuation and broadening of host or cell-type ranges [27, 28]. This protein has also been reported as a determinant of cell tropism in IBV, as some amino acids contained in S protein contribute to attenuation of IBV [29]. Variations in the S1 gene may reduce the ability of CK/CH/LDL/97I P115 to infect host cells after serial passage in eggs, but not its ability to infect the choioallantoic membrane, where IBV replication initially occurs in embryonated chicken eggs. However, such conclusions should be drawn with caution, as the results of this and other studies found no identical substitutions, insertions, or deletions in S proteins between different pathogenic IBV strains and their embryo-passaged, attenuated derivatives, even though the attenuated IBV strains showed distinctly different biological features, including lack of pathogenicity to 1-day-old SPF chicks compared to the pathogenic parental strains [26, 30, 31]. Some findings suggested that the replicase gene, rather than the S1 or S gene, may be responsible for viral pathogenicity [32, 33].

The S2 subunit of the spike protein of CoVs associates non-covalently with the S1 subunit and contains the transmembrane and C-terminal cytoplasmic tail domains. The ectodomain region [33] and two heptad repeat regions [34] of subunit S2 are involved in oligomerization of the S protein and required for entry into susceptible cells [35–37]. Changes in the S2 gene may affect viral entry [32]. In this study, multiple changes in the S2 gene occurred during passage of CK/CH/LDL/97I in embryonated eggs, which may have had an effect on viral entry into chicken host cells in vivo, indirectly decreasing the replication capacity. However, introduction of an S protein from a virulent IBV isolate did not confer virulence to the apathogenic strain Beau-R [29]. A further study showed that replacing all the Beaudette structural and accessory proteins with those from a virulent IBV isolate did not restore virulence [45]. These results suggest that IBV attenuation may not be caused by changes in the S2 gene. However, it has been speculated that changes in the S2 protein may contribute to adaptation of the field virus to chick embryonic tissue [32].

As in other CoVs, the IBV E protein is a small structural protein containing a single hydrophobic domain with multiple functions [38–42]. Although information on the IBV E protein is limited [41, 43], those from different CoVs perform similar functions during viral infection [44]. In the current study, we identified a mutation of nucleotide 184 (C-T) in P115, compared to P5, resulting in an amino acid substitution (Ala→Ser). However, this substitution may have only a minor effect on viral replication after 115 passages in eggs because only the hydrophobic domain (amino acids 12–32) of IBV E is important for efficient viral replication after 115 passages in eggs because only the hydrophobic domain (amino acids 12–32) of IBV E is important for efficient viral replication [41]. The cytoplasmic tail of IBV E is responsible for its interaction with IBV M [4], and mutations in the cytoplasmic tail of mouse hepatitis virus (MHV) E by targeted RNA recombination resulted in the production of elongated virions [45]. Therefore, substitutions in the cytoplasmic tail of the IBV P115 E protein caused by serial passages warrant further investigations.

No previous studies to date have investigated the function of the IBV intergenic UTR between M and gene 5, though some sequence diversities and mutations have been found in this region among IBV strains [32, 46]. This region contains a potential ORF with an initiation
codon immediately downstream of the M gene stop codon in some IBV strains. This study identified a similar 273-nucleotide ORF that potentially encodes a 90-amino acid product in CK/CH/LDL/97I P5 at a similar position; however, this ORF was extended in P115 by two nucleotide insertions, resulting in an ORF of 285 nucleotides that potentially encodes a 11-kD, 94-amino acid product. We could not predict the functions of the ORF in CK/CH/LDL/97I P5 or the potential effect of the insertions. The lack of subgenomic mRNA for this potential ORF, the long distance between the initiation codon and potential transcription regulatory sequence, and the loss of the potential ORF in some IBV strains indicate that this ORF is probably a pseudogene [32]. Furthermore, removal of the intergenic UTR from an apathogenic IBV had no effect on the functionality of the virus [32].

This study detected a substitution (Tyr→The) in the N protein due to the mutation at nucleotide position 398, between the P5 and P115 viruses. The N protein binds to the 3′ end of the UTR, which is essential for the synthesis of negative-strand viral RNA. The change in the N gene suggests that it may have an impact on viral replication and thus on viral pathogenesis, although the sequence-specific interaction between the N gene and 3′-UTR remains unclear. The amino acid substitution at residue 188 (Thr→Ile/Ala) in the M protein, which was related to antigenicity and/or virulence in IBV strains H52/H120, TW2296/95, and Arkansas [30], was not found in our CK/CH/LDL/97I strain; however, a nucleotide substitution between P5 and P115 was detected that resulted in an amino acid substitution at residue 223 (Gly→Ser). This substitution may affect the interaction between M and the cytoplasmic tail of IBV E [4], and thereby viral budding.

The IBV replicase gene encodes 15 nsps, some with known enzymatic functions [47]. However, the functions of these proteins in the context of pathogenesis remain poorly understood, although some nsps of other CoVs have been linked to the loss of virulence [32]. Like its homologs in other CoVs, 3CLpro of IBV is encoded by ORF1a and resides in nsp5. This protease specifically cleaves polyproteins 1a and 1ab at 11 sites to produce 12 mature products (nsp5–nsp16). A cluster of small nsps, including nsp6, nsp7, nsp8, nsp9, and nsp10, is located at the C-terminal region of the 1α polyprotein. Although the functional roles of these proteins in IBV are unknown, roles for these nsps in RNA replication and transcription of other CoVs are emerging from biochemical and structural analyses [48]. In the current study, we found no differences in nucleotide sequences between CK/CH/LDL/97I P5 and P115 in nsp5, nsp6, nsp7, nsp8, or nsp10. Other groups have attenuated the virulent Ark DPI 11 strain of IBV following 101 passages in embryonated eggs (Ark DPI 101) and compared the complete genomes of both viruses. They identified two and one amino acid substitutions in nsp6 and nsp10, respectively, but none in nsp5, nsp7, or nsp8 [47], as in the present study. In addition, although substitutions were found in nsp4, nsp9, nsp11/12, nsp14, nsp15, and nsp16 in our study, these substitutions did not produce amino acid substitutions due to viral subpopulation selection, other than several mutations.

In contrast, apart from alterations due to viral subpopulations, three, two, and one amino acid substitutions were found in nsp1/2, nsp3, and nsp13 proteins, respectively, between P5 and P115. Of the three amino acid substitutions, one similar substitution was also reported in this coding region between challenge and vaccine M41 strain, and Ark DPI 11 and attenuated DPI 101 [47]. A recent report showed that nsp2 plays a role in facilitating de novo IBV protein synthesis by blocking protein kinase R phosphorylation of eukaryotic initiation factor 2 (eIF-2α), which shuts down protein synthesis. In addition, nsp2 induces expression of GADD34, which dephosphorylates eIF-2α [49]. The nsp3 of CoV contains multiple domains including an acidic domain, an ADP-ribose-1′′-phosphatase, a papain-like proteinase (PL2pro), a Y domain, and a transmembrane domain [50]. Inactivation of ADP-ribose-1′′-phosphatase activity in MHV nsp3 reduced viral replication in the livers of infected mice, but did not induce liver disease [51]. Two amino acid substitutions in this study were located in the viral proteinase PL2pro and Y domains, respectively, which were similar to the positions altered between Ark DPI 11 and 101 [47]. The substitution in PL2pro may restrict viral maturation or replication. However, we were unable to predict the effects of the Y-domain change because the role of this domain in IBV is currently unknown. nsp13 contains an RNA helicase and has an important function in viral replication. An amino acid substitution between CK/CH/LDL/97I P5 and P115 was found in the helicase domain at nucleotide position 15859, resulting in an amino acid substitution (Leu→Pro). This substitution was similar to that in the helicase domain of Ark PDI, located at nt 15763. This substitution in the attenuated P115 strain might alter viral replication [47].

The IBV replicase gene is not usually associated with antigenicity, immunogenicity, or tissue tropism. The amino acid substitutions in the ORF1a/1b proteins offer insight into putative residues that may be involved in the adaptation of chick embryonic tissue and subsequent at-
tenuation of the virus. It is possible that very few nucleotide substitutions within the replicase gene may cause attenuation following serial passage in embryonated eggs [32, 47], although the role of the replicase gene in pathogenicity is not well understood.

The 5′- and 3′-UTRs in the genome of CoVs, like most RNA viruses, usually harbor important structural elements involved in replication and/or translation [7–11]. In the present study, we found two and three variations in the 5′- and 3′-UTRs of the attenuated CK/CH/LDL/97I P115 virus, respectively, compared to the pathogenic P5 parental strain. These nucleotide variations may play a role in pathogenicity by affecting the secondary structures of cis-acting elements involved in viral replication [52].

Selection of a more fit virus subpopulation and mutations in the S1 gene have reportedly been associated with the replication of modified live IBV vaccine viruses in chickens [53]. S1 gene sequence analysis demonstrated that distinct virus subpopulations were selected when chickens were vaccinated with live-attenuated IBV strains [21]. Further analysis confirmed that the virus subpopulations differed between microenvironments in distinct host tissues [54]. This investigation did not compare the replication capacities of CK/CH/LDL/97I before and after serial embryo passage in the oviduct of female chickens, or assess damage to the reproductive tract, egg production, or egg quality in laying chickens. However, IBV replication in the epithelium of the oviduct has been shown to result in decreased egg production and quality [55]. The damage is more severe and permanent if young chicks are affected [56], although repeated embryo passage was shown to reduce the virulence of some IBVs in the chicken oviduct [57]. Limited information is currently available regarding the pathogenicity of CK/CH/LDL/97I-type IBV strains and embryo-passaged, attenuated P115 in the chicken oviduct. Future studies will therefore focus on the effects of these IBV strains on the chicken oviduct.

Compared with the large genetic differences between cell-adapted CoVs and their parental viruses [58, 59], few genetic changes were found between the embryo-passaged, attenuated CoVs and their pathogenic parental viruses [15, 16, 47]. The result is still very interesting to understanding of molecular markers of IBV attenuation, tissue tropism, and replication capacity changes, as in the case of the transmissible gastroenteritis virus, which showed that two amino acid substitutions at the N-terminus of spike protein resulted in loss of enteric tropism [60]. The current sequence analyses were unable to identify specific amino acid residues responsible for these changes because the attenuated strain differed from the parental strain at multiple positions. Further investigations using reverse genetics and animal studies are therefore necessary to verify the exact functions of these changes. In addition, none of the sequence substitutions were shared by all pathogenic IBV strains or their attenuated derivatives [26, 30, 31, 47], indicating changes in viral replication, pathogenicity, antigenicity, immunogenicity, or tissue tropism might be attributable to different genes in the viral genome. However, some of the sequence substitutions identified in this study could be used as the potential molecular markers, at least between the CK/CH/LDL/97I-type pathogenic and attenuated strains, providing important information for the development of specific diagnostics of differentiating CK/CH/LDL/97I-type IBV infections from vaccination because the attenuated P115 can be used as a potential vaccine candidate [20] to control the circulation of CK/CH/LDL/97I-type IBVs in China and regions of the Middle East in recent years [18, 19, 21, 23].

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References

1 Carstens EB: Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses. Arch Virol 2012;157:1411–1422.
2 Cavanagh D, Gelb J: Infectious bronchitis; in Saif YM (ed): Diseases of Poultry, ed 12. Ames, Wiley-Blackwell Publishing, 2008, pp 101–120.
3 Cavanagh D: Coronavirus avian infectious bronchitis virus. Vet Res 2007;38:281–297.
4 Boursnell ME, Brown TD, Foulsh IJ, Green PF, Tomley FM, Binns MM: Completion of the sequence of the genome of the coronavirus avian infectious bronchitis virus. J Gen Virol 1987;68:57–77.
5 Dufour D, Mateos-Gomez PA, Enjuanes L, Gallego J, Sola I: Structure and functional relevance of a transcription-regulating sequence involved in coronavirus discontinuous RNA synthesis. J Virol 2011;85:4963–4973.
Pasternak AO, Spana WJ, Snijder EJ: Nidovirus transcription: how to make sense...? J Gen Virol 2006;87:1403–1421.

Chang KY, Hofmann MA, Sethna PB, Brian DA: A cis-acting function for the coronavirus leader in defective interfering RNA replication. J Virol 1994;68:8223–8231.

Raman S, Bouma P, Williams GD, Brian DA: Stem-loop III in the 5′ untranslated region is a cis-acting element in bovine coronavirus defective interfering RNA replication. J Virol 2003;77:6720–6730.

Raman S, Brian DA: Stem–loop IV in the 5′ untranslated region is a cis-acting element in bovine coronavirus defective interfering RNA replication. J Virol 2005;79:12434–12446.

Goebel SSJ, Miller TB, Bennett CJ, Bernard KA, Masters PS: A hypervariable region within the 3′ cis-acting element of the murine coronavirus genome is nonessential for RNA synthesis but affects pathogenesis. J Virol 2007;81:1274–1287.

Züst R, Miller TB, Goebel SSJ, Bernard KA, Masters PS: Genetic interactions between an essential 3′ cis-acting RNA pseudoknot, replicate gene products, and the extreme 3′ end of the mouse coronavirus genome. J Virol 2008;82:1214–1228.

Welsh MJ Jr, Cloud SS: Effect of serial embryo passage of an Arkansas-type avian infectious bronchitis virus isolate on clinical response, virus recovery, and immunity. Avian Dis 1983;27:679–687.

Jackwood MW, Hilt DA, Brown TP: Attenuation, safety, and efficacy of an infectious bronchitis virus G989 serotype vaccine. Avian Dis 2003;47:627–632.

Bijlenga G, Cook JKA, Gelb J Jr, de Wit JJ: Development and use of the H strain of avian infectious bronchitis virus from the Netherlands as a vaccine. Avian Pathol 2004;33:550–557.

Huang Y-P, Wang C-H: Development of attenuated vaccines from Taiwanese infectious bronchitis virus strains. Vaccine 2006;24:785–791.

Gelb J Jr, Cloud SS: Effect of serial embryo passage of an Arkansas-type avian infectious bronchitis virus isolate on clinical response, virus recovery, and immunity. Avian Dis 1983;27:679–687.

Gelb J Jr, Cloud SS: Effect of serial embryo passage of an Arkansas-type avian infectious bronchitis virus isolate on clinical response, virus recovery, and immunity. Avian Dis 1983;27:679–687.

Kuo SM, Wang CH, Hou MH, Huang YP, Kao HW, Su HL: Identification of a new region of an avian infectious bronchitis coronavirus strain after serial passage in embryos. Vaccine 2009;27:4630–4640.

Gellhausen HE, Ligon FB, Lukert PD: The molecular epidemiology of infectious bronchitis virus isolates from China and Southeast Asia. Avian Dis 2001;45:201–209.

Schulze B, Cavanagh D, Herrler G: Neuraminidase treatment of avian infectious bronchitis virus spike proteins to host factors reflects virus tropism and pathogenicity. J Virol 2011;85:8903–8912.

Cavanagh D, Picault JP, Gough RE, Hess M, Mawditt KL, Britton P: Variations in the spike protein of the 793/B type of infectious bronchitis virus in the field and during alternate passage in chickens and embryonated eggs. Avian Pathol 2005;34:20–25.

Schickli JH, Zelus BD, Wentworth DE, Savicki SG, Holmes KV: The murine coronavirus mouse hepatitis virus strain A59 from persistently infected murine cells exhibits an extended host range. J Virol 1997;71:1999–9507.

Baric RS, Sullivan E, Hensley L, Yount B, Chen W: Persistent infection promotes cross-species transmissibility of mouse hepatitis virus. J Virol 1999;73:638–649.

Casais R, Dove B, Cavanagh D, Britton P: Reassortant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. J Virol 2003;77:9084–9089.

Huang YP, Wang CH: Sequence changes of infectious bronchitis virus isolates in the 5′-3.7 kb of the genome after attenuating passage in embryonated eggs. Avian Pathol 2007;36:59–67.

Lee HJ, Youn HN, Kwon JS, Lee YJ, Kim JH, Lee JB, Park SY, Choi IS, Song CS: Characterization of a novel live attenuated infectious bronchitis virus vaccine candidate derived from a Korean nephropathogenic strain. Vaccine 2010;28:2887–2894.

Armesto M, Cavanagh D, Britton P: The replicative gene of avian infectious bronchitis virus is a determinant of pathogenicity. PLoS One 2009;4:e7384.

Luo ZL, Weiss SR: Roles in cell-to-cell fusion of two conserved hydrophobic regions in the coronavirus spike protein. Virology 1998;244:483–494.

De Groot RJ, Luitjes W, Horzinek MC, van der Zeijst BAM, Spana WJ: Evidence for a coiled-coil structure in the spike proteins of coronaviruses. J Mol Biol 1987;196:963–966.

Tripathi B, Howard MW, Jobling M, Holmes RK, Holmes KV: Structural characterization of the SARS-coronavirus spike S fusion protein. J Virol Chem 2004;27:20836–20849.

Kuo L, Masters PS: The small envelope protein E is not essential for murine coronavirus replication. J Virol 2003;77:4597–4608.

Kuo L, Masters PS: The small envelope protein E is not essential for murine coronavirus replication. J Virol 2003;77:4597–4608.
48 Johnson MA, Jaudzems K, Wuthrich K: NMR structure of the SARS-CoV nonstructural protein 7 in solution at pH 6.5. J Mol Biol 2010;402:619–628.

49 Wang X, Liao Y, Yap PL, Png KJ, Tam JP, Liu DX: Inhibition of protein kinase R activation and upregulation of GADD34 expression play a synergistic role in facilitating coronavirus replication by maintaining de novo protein synthesis in virus-infected cells. J Virol 2009;83:12462–12472.

50 Imbert I, Snijder EJ, Dimitrova M, Guillemot JC, Leclerc P, Canard B: The SARS-coronavirus PL nc domain of nsp3 as a replication/transcription scaffolding protein. Virus Res 2008;133:136–148.

51 Eriksson KK, Cervantes-Barragan L, Ludewig B, Thiel V: Mouse hepatitis virus liver pathology is dependent on ADP-ribose-1′-phosphatase, a viral function conserved in the alpha-like supergroup. J Virol 2008;82:12325–12334.

52 Williams M, Chang RY, Brian DA: Evidence for a pseudoknot in the 3′ untranslated regions of the bovine coronavirus genome. Adv Exp Med Biol 1995;380:511–514.

53 McKinley ET, Hilt DA, Jackwood MW: Avian coronavirus infectious bronchitis attenuated live vaccines undergo selection of subpopulations and mutations following vaccination. Vaccine 2008;26:1274–1284.

54 Gallardo RA, van Santen VL, Toro H: Host intraspatial selection of infectious bronchitis virus. Avian Dis 2008;54:807–813.

55 Sevoian M, Levine PP: Effects of infectious bronchitis on the reproductive tracts, egg production, and egg quality of laying chickens. Avian Dis 1957;1:136–164.

56 Crinion RAP, Ball RA, Hofstad MS: Pathogenesis of oviduct lesions in immature chickens following exposure to infectious bronchitis virus at one day old. Avian Dis 1971;15:32–41.

57 Crinion RAP, Hofstad MS: Pathogenesis of two embryo passage levels of avian infectious bronchitis virus for the oviduct of young chickens of various ages. Avian Dis 1983;27:679–687.

58 Fang SG, Shen S, Tay FP, Liu DX: Selection of and recombination between minor variants lead to the adaptation of an avian coronavirus to primate cells. Biochem Biophys Res Commun 2005;336:417–423.

59 Zhang X, Hasoksz M, Spiro D, Halpin R, Wang S, Vlasova A, Janies D, Jones LR, Ghe din E, Saif LJ: Quasispecies of bovine enteric and respiratory coronaviruses based on complete genome sequences and genetic changes after tissue culture adaptation. Virology 2007;363:1–10.

60 Ballesteros ML, Sanchez CM, Enjuanes L: Two amino acid changes at the N-terminus of transmissible gastroenteritis coronavirus spike protein result in the loss of enteric tropism. Virology 1997;227:378–388.