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Experimental confirmation of recombination upstream of the S1 hypervariable region of infectious bronchitis virus

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Abstract

Chimeric infectious bronchitis virus (IBV) genomes with cross-over sites in the S1 gene were generated by co-infection with two distinct IBV strains. Recombinant viruses were collected from chicken embryos, embryonic cultured cells and chickens co-infected with Ark99 and Mass41 strains and purified by differential centrifugation. The recombinant S1 genes were identified by reverse transcription polymerase chain reaction (RT-PCR) using heterologous primers and confirmed by nucleotide sequencing. The recombinants with Ark99 5% and Mass41 3% sequences were identified following the in vitro, in ovo and in vivo co-infections. Mixed RNA extracted from Ark99 and Mass41 did not produce RTPCR products with these primers at the PCR conditions used. Cross-over sites within the amplified 580 (Mass41) or 604 (Ark99) bases of the 5% S1 gene could only be detected between nucleotides 50 and 155. While this region, lying upstream of the S1 hypervariable region, corresponded with sites commonly identified in naturally occurring isolates, recombination sites identified in these studies could not be detected within the HVR of S1 of the genomes of chimeric viruses. © 1997 Elsevier Science B.V.

Keywords: Coronavirus; Infectious bronchitis virus; Recombination; Co-infection

Infectious bronchitis virus (IBV), with large positive-sense RNA genome of 27.6 kb, is a prototype virus of the Coronaviridae family (Boursnell et al., 1987; Collisson et al., 1992). IBV is an agriculturally important pathogen of poultry and despite routine use of vaccines, variant stains continue to cause outbreaks in field situations (personal observations, King and Cavanagh, 1991; Wang et al., 1993). The continuing emergence of new strains may be explained in part by the apparent common occurrence in nature of recombination between IBV strains (Wang et al., 1993, 1994).

Both in vitro and in vivo RNA recombination between mouse coronavirus genomes in the presence and absence of selective biological pressure has been experimentally demonstrated (Lai et al.,...
The glycosylated S protein, a major structural protein of IBV, is posttranslationally cleaved into S1 and S2. The S1 protein has been shown to be responsible for attachment to cellular host membranes (Collisson et al., 1992), and for the induction of neutralizing and hemagglutination inhibiting antibodies (Koch et al., 1992; Kant et al., 1992). We have recently also demonstrated that the S1 can induce IBV specific CTL responses in the chick (Seo and Collisson, manuscript in preparation). Comparisons of the S1 from a number of distinct IBV strains have identified a hypervariable region (HVR) between 50–150 amino acids from the amino terminus (Cavanagh et al., 1988; Kusters et al., 1989; Wang et al., 1994).

We have shown that sequences lying within the S1 of the spike of IBV, upstream of the HVR, are a common site for major shifts in sequence homology in naturally occurring strains (Wang et al., 1993, 1994). This frequently used cross-over site immediately adjacent to the HVR included up to 153 bases at the 5′ end of the S1 gene (Wang et al., 1994). Whereas variations within the S1 correlates with serotype, the HVR is thought to be closely associated with major neutralization epitopes (Neister et al., 1987; Lenstra et al., 1989; Ignjatovic and Galli, 1994). Recombination in the IBV genome has recently been experimentally demonstrated in the N gene following co-infection of embryonated chicken eggs (ECE) infected with the closely related Mass41 and Beaudette strains (Kottier et al., 1995).

The following experiments were designed to identify experimentally generated chimeric viruses with cross-over sites lying in the first 560 or 580 bases (Mass41 or Ark99 strains, respectively) of the S1 gene, which includes the identified commonly used cross-over region and the HVR. The Mass41 and Ark99 strains were used as parental strains for co-infection because they have extensive differences in the nucleotide sequences of the S1 HVR, have been implicated in the generation of field isolates, and have a number of nucleotide differences within the flanking regions of the targeted sequences (Wang et al., 1993, 1994).

Cultured chicken embryonic kidney (CEK) cells were co-infected with CEK cell adapted Mass41 and Ark99 strains. The CEK cells were prepared in our laboratory from 19 day-old chicken embryos (Churchill, 1965). Monolayers of confluent CEK grown in 25 cm² flasks were inoculated simultaneously or singly with 10⁶.0 EID₅₀ of Mass41 and 10⁶.0 EID₅₀ of Ark99 viruses. Progeny viral particles used to detect recombination were harvested 48 h post-infection (pi). Supernatants were collected following repeated freeze-thaw cycles of infected cells and viral particles were concentrated after clarifying the supernatant at 8000 × g for 30 min by centrifuging at 100 000 × g for 1 h (Williams et al., 1992). The viral RNA was extracted using the proteinase K/chloroform/phenol method (Sambrook et al., 1989) and reverse transcription of RNA was initiated using the downstream strain specific primers described in Table 1 for either Ark99 and Mass41 viruses and Superscript II reverse transcriptase (Gibco-BRL) (Wang et al., 1993). Following the removal of RNA from the hybrids of RNA and newly synthesized first strand cDNA with RNaseH, the remaining single-stranded RNA was amplified by the polymerase chain reaction (PCR) using the primers described in Table 1.

| Reference | Nucleotide sequence | Location from S1 start codon |
|-----------|--------------------|-----------------------------|
| Ark-U     | CTAAGGAACG-GTAAATGTGTA | −20 |
| Mass-U    | ATAGTGTGGT-AAATCCTGG | −20 |
| Ark-D     | CTA-CAACATC-TTCAGTATAG | 580 |
| Mass-D    | ATGTAACATC-GTGGTCTCA | 604 |

*U refers to upstream primers.

*D refers to downstream primers.
cDNA was used as template for PCR with homologous and/or heterologous primers. Amplified PCR products were observed by agarose gel electrophoresis (Fig. 1). Chimeric S1 could be identified by agarose gel electrophoresis of RTPCR amplified products generated with strain specific heterologous primers.

The agarose gel separated amplified cDNA from infected and uninfected CEK cells are shown in Fig. 1a. Similar results were obtained with chicken embryonic lung cells (data not shown). The products from control experiments showed that homologous primers could amplify viral cDNA of the predicted size from particles isolated from either Ark99 or Mass41 infected cells. No recombinant RTPCR product was detected using heterologous primers with either Mass41 or Ark viral RNA, or with mixture of Ark and Mass41 viral RNAs. Nor was any RTPCR product generated from uninfected cellular RNA using both Ark99 and Mass41 specific primers. However, RTPCR amplified recombinant cDNA products could be detected from the viral RNA of Mass and Ark co-infected CEK cells using both sets of heterologous primers. The amounts of viral RNA used for RNA extraction in the experimental and control groups were equivalent.

Recombination was also evaluated in embryonating chicken eggs (ECE). Allantoic sacs of 11-day old ECE were inoculated with about 10^5.7 EID_50 of ECE adapted Ark99 and Mass41 viruses. The allantoic fluids from infected ECE were harvested 48 h pi and concentrated by differential centrifugation as described above (Williams et al., 1992). Recombinant RTPCR products were clearly detected following amplification with the Ark-U and Mass-D primer set (Fig. 1b). No recombinant RTPCR product was observed with RNA from the uninfected embryos.
Recombination was confirmed and the approximate cross-over sites were identified by cloning and sequencing of cDNA generated from the Ark-U and M41-D primers which consistently produced a product from in vitro or in ovo infection that could be easily seen on an agarose gel (Fig. 2). RTPCR generated cDNA bands with the predicted 600 nt size were excised from agarose gels and the DNA extracted with GeneClean (Biot101, Vista, CA). The DNA was sequenced after cloning into a PCR cloning vector (Invitrogen, San Diego, CA). The sequence information for the recombinant viruses were compared with the published sequences of the parental strains (Binns et al., 1985; Wang et al., 1993). Cross-over sites in the in vitro generated recombinant viruses were detected within cDNA corresponding to nucleotides 50–60 and nucleotides 95–100 of the S1 gene. Recombination in the chimeric RNA derived from in ovo co-infection occurred at about nucleotide 99. These sites lie within the putative recombination ‘hot spot’ indicated from sequencing of the S1 of a number of natural isolates (Wang et al., 1993, 1994). No shifts in homology could be detected within the HVR which extends from nucleotides 160–560. An identical change at nucleotide 52–55 (TGCT to CTTG) from our original Ark sequence or the...
Mass41 sequence occurred in three chimeric molecules (Binns et al., 1985; Wang et al., 1993). This change may reflect mutations in our stocks.

In order to reproduce recombination in the more natural in vivo setting, chicks were also co-infected with Mass41 and Ark99 viruses. Two-day old specific pathogen-free chicks were inoculated intranasally/intraorbitally with 500 times the recommended dose of both Mass41 and Ark99 vaccine strains. The infected chicks were sacrificed by cervical dislocation six days pi. Five of seven chicks co-infected with 500 doses suffered severe respiratory distress with sneezing, labored breathing, and rales, and two of the five succumbed three and five days pi. Lungs were collected, minced and homogenized in Eagle’s MEM (Gibco, BRL, Bethesda, MD) and the viruses were collected from the supernatant as described above. The RNA of viruses from the lungs of one chick that died, and three sick and two healthy chicks were extracted for RTPCR using the Ark-U and Mass-D primer set. Fig. 1c shows the agarose gel with amplified chimeric cDNA products of RNA from the lungs of one co-infected sick chick that died, and two healthy chicks (Fig. 1c). No products were detected from the lungs of the remaining infected chicks nor uninfected chicks.

cDNA clones were generated from the RTPCR products amplified from the chick that died (C-1) and one clinically healthy chick (C-3). Nucleotide sequencing of the viral RNA from the in vivo co-infected lungs indicated that cross-over sites of recombination for both RNA were at approximately nucleotide 155 of the S1 gene, a region immediately upstream of the HVR. Again, no cross-over sites were identified within the HVR (Fig. 2).

Recombination within the S1 gene was experimentally produced from genomes within viral particles rather than cell lysates. Although the viability of the recombinant viruses was not demonstrated in this study, the experimentally produced S1 gene recombination events resembled the shifts in homology observed in nature (Wang et al., 1993, 1994). The approximate locations of all cross-over sites within chimeric RNA were located between nucleotides 50 and 155 (Fig. 2).

Comparisons of sequences from the naturally occurring IBV strains had indicated that cross-over sites were concentrated at the 5' end of the S1 genes (Wang et al., 1994; unpublished data). A second, less often used site was found close to the 3’ end of S1 (Wang et al., 1993, 1994). Although frequencies of recombination are not known, the fact that the HVR and adjacent downstream 600 or more nucleotides may seldom be targets for cross-over events suggests that the region lying upstream, in fact, is more susceptible for recombination. This region may have structures or sequences that promote polymerase interchange of templates. However, because the additional sequences examined were within the HVR, it is also possible that HVR sequences are not favorable sites for recombination. During the process of synthesizing potential chimeric molecules, the variability of the HVR may prohibit necessary RNA-RNA interactions. For example, released, replicating S1 HVR may not be capable of binding to an HVR of new template. An alternative explanation for the absence of detectable recombination events is that chimeric HVR may be less likely to express functional S1 required for viable virus than other chimeric regions, for example, the initial 155 bases of the S1.

The Ark99 and Mass41 strains were chosen because they are most often used in the USA for vaccination programs and consequently, are artificially maintained in the poultry population. The Mass serotype vaccines have historically by far been most often used to induce flock immunity and does demonstrate more cross-reactivity with other IBV strains (Sneed et al., 1989). Because the parental sequences of many recombinant, naturally occurring strains are derived from distinct Mass41-like sequences, it is a concern that the vaccine may be contributing to the evolution of new strains (Wang et al., 1994). These studies confirm that, with excessive doses of parental virus used to accelerate the rate of recombination, RNA from a Mass serotype can participate in cross-over events resulting in progeny with sequences of the Mass origin and sequences derived from a second parent with the Ark serotype. These studies, which were not designed to examine virulence of experimentally produced recombi-
nants, did produce a chimeric S1 from a sick chick and one from a healthy chick. The contribution, if any, of these recombination events to the generation of virulent strains can only be determined with further investigations.

The effect of recombination on immunologic evasion in the vaccinated chick is also not known. A successful escape mutant must certainly be viable, if not virulent, and have altered critical epitopes in order to cause disease in an immunized host. Preliminary studies in which recombinant molecules were detected following transfection of in vitro transcribed RNA from the Gray strain into Mass41 infected embryonic cultured cells have suggested that targeted recombination is also feasible (unpublished data). The capacity for Mass41 strains and Mass vaccines, in particular, to participate in the occurrence of new isolates and the ready experimental generation of Mass41 recombinants in vivo, as well as in vitro and in ovo, may further raise concern about the routine use of live IBV to control disease. Caution should also be encouraged in the use of attenuated vaccines of any virus that can readily undergo recombination.

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