FIRST EXPERIMENTAL EVIDENCE OF RECOMBINATION IN INFECTIOUS BRONCHITIS VIRUS

Recombination in IBV

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ABSTRACT

A high frequency of recombination has been shown to occur during replication of the coronavirus mouse hepatitis virus (MHV) *in vitro* as well as *in vivo*. Although sequencing of field strains of coronavirus infectious bronchitis virus (IBV) has indicated that IBV strains also undergo recombination, there has been no experimental evidence to support this deduction. To investigate whether recombination occurs in IBV, embryonated eggs were coinfected with IBV-Beaudette and IBV-M41. Potential recombinants were detected by strain-specific polymerase chain reaction (PCR) amplifications, using oligonucleotides corresponding to regions in the 3′ end of the genome. Sequencing of the PCR products confirmed that a number of recombinations had occurred between the two strains.

INTRODUCTION

RNA-RNA recombination involves the exchange of genetic information between non-segmented viruses and has been detected at a high frequency in picornaviruses and in coronavirus MHV. In MHV, recombination sites were observed over almost the entire genome and several recombinants resulted from multiple cross-over events (1). A recombination map for MHV (2) suggested the frequency of recombination to be roughly 1% per 1300 nucleotides, which represents 25% for the entire genome, assuming that recombination occurs randomly. Recombination has also been shown to occur between coronavirus

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genomic RNA and transfected RNA, enabling site-specific mutagenesis (3,4,5). However, such recombinants have only been sought and detected in the 5′ and the 3′ ends of the genome.

Studies on the mechanisms of recombination in picornaviruses showed that recombination was more likely to occur by template-switching than by breaking and rejoining of RNA and recombination frequencies were found to be higher between more closely related strains (6). The mechanism of recombination in coronaviruses has been suggested to occur by a mechanism similar to that described for poliovirus (7). Although these findings have contributed greatly to the understanding of RNA recombination in viruses, MHV remains the only coronavirus in which recombination has been experimentally demonstrated. Sequencing studies on isolates of IBV have suggested that recombination does occur in the field (8,9,10) but there is no direct evidence of recombination in coronaviruses other than MHV.

In this paper recombination was tested in the coronavirus IBV using embryonated eggs, coinfected with IBV-M41 and IBV-Beaudette. IBV-M41 and IBV-Beaudette are both of the Massachusetts serotype. The entire genome of Beaudette has been sequenced (11) and several regions of M41 have been sequenced (as reviewed in 12). Beaudette has 184 nucleotides inserted in the noncoding region at the 3′ end of the genome when compared with M41. Otherwise, the two strains have a high degree of sequence identity, increasing the chance of recombination between the two strains. Strain-specific oligonucleotides were designed, from sequences within the 3′ ends of both IBV strains, for detection of potential recombinants. Potential recombinants were detected by RT-PCR using a negative sense Beaudette-specific primer and a positive sense M41-specific primer or vice versa. PCR products from potential recombinants were sequenced and analyzed.

METHODS

Coinfection of Embryos with IBV M41 and Beaudette

Eleven-day-old specified pathogen free embryonated Rhode Island Red (RIR) eggs were inoculated, in quintuplicate, in the allantoic fluid, with $7X10^6$ egg infectious dose $_{50}$ (EID_{50}) IBV-Beaudette, or with $3X10^7$ EID_{50} IBV-M41, or coinfected with $7X10^6$ EID_{50} IBV-Beaudette and $3X10^7$ EID_{50} IBV-M41 (inoculum X1), or with $7X10^6$ EID_{50} IBV-Beaudette and $3X10^6$ EID_{50} IBV-M41 (inoculum X2), or with $7X10^6$ EID_{50} IBV-Beaudette and $3X10^5$ EID_{50} M41 (inoculum X3) or inoculated with PBSa. Eggs were sealed with collodium and incubated at 37°C for 24 h. After overnight incubation at 4°C the allantoic fluid was harvested.

Reverse Transcription (RT)

Virions were pelleted from the allantoic fluid and RNA was extracted from virions, using the guanidinium isothiocynate method (13). The final pellets were dissolved in 50 µl of nuclease-free water (Sigma). For first strand cDNA synthesis 3 µl of the extracted RNA was denatured for 10 min at 60°C in the presence of 0.21 nmol of oligonucleotide 100 (CAGGATATCGCTCTAACTCTATACTAGCCT), complementary to position 27587-27607 at the 3′ end of the IBV-genome. The mixture was immediately cooled on ice and incubated for 2 h at 42°C in a 35 µl reaction mixture containing; 250 U of M-MLV reverse transcriptase (Promega), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 7 mM DTT and 1.5 mM of dNTPs.

Polymerase Chain Reaction (PCR)

From the first strand cDNA reaction mixture, 5 µl was taken and mixed in a total volume of 50 µl containing, 1 5 U Taq DNA polymerase, 50 mM KCl, 10 mM Tris-HCl (pH 9 0), 0 1% Triton-X100, 1 5 mM $MgCl_2$, 0 2 mM dNTPs and 0 3 nmol of positive and negative sense primers (see results) The mixture was overlayered with 50 µl of mineral oil and subjected to 25 cycles on a Hybaid Omnigene heating block at 94°C for 1 min, 42°C for 2 min and 72°C for 3 min, followed by a 9 min elongation step at 72°C

Cloning and sEquencing

PCR products, derived from potential recombinants, were cloned into the pCR™ vector (Invitrogen) according to the manufacturer's protocol and sequenced on a 373A DNA Sequencer (Applied Biosystems Inc) using the PRISM™ Ready Reaction DyeDeoxy Terminator cycle sequencing kit according to the manufacturer's instructions (Applied Biosystems Inc)

RESULTS

Strain-specific PCR has been demonstrated to be a powerful technique for studying RNA recombination in the absence of selection pressure (14, 15) Strain-specific primers were designed from the 3′ end of the genomes of IBV-Beaudette and M41 with as many nucleotide differences as possible, at the 3′ end of the oligonucleotides (Fig 1) Recombination was detected in MHV within a region of 330 nucleotides (16),therefore IBV strain-specific primers over 300 nucleotides apart from each other were chosen The IBV-Beaudette specific primers were of positive sense, primer B5 (CCTGATAACGAAAACATT), corresponding to position 26041-26058 and of negative sense, primer B7 (GCACAGCAACAATACAAT), complementary to position 27233-27250 of the IBV-Beaudette genome The IBV-M41 specific primers were of positive sense, primer M6 (CCTGATAATGAAAATCTA), corresponding to position 26041-26058 and of negative sense, primer M8 (AGGTCAATGCTTTATCCA), complementary to position 26990-2707 The nucleotides positions in M41 referred to equivalent regions on the IBV-Beaudette genome

RT-PCR was carried out on virion RNA obtained from the infected eggs as described in the methods As a control to show that no PCR artifacts were produced under the chosen conditions, equal amounts of RNA from eggs infected only with Beaudette and only with M41 were mixed for one of the RT-PCR amplifications PCR amplifications were carried out on all cDNA samples with the Beaudette-specific primers, B5 and B7 (Fig 2A), the M41-specific primers, M6 and M8 (Fig 2B) and to detect potential recombinants, with the primer combinations, B5 and M8 (Fig 2C) and M6 and B7 (Fig 2D) No PCR products were

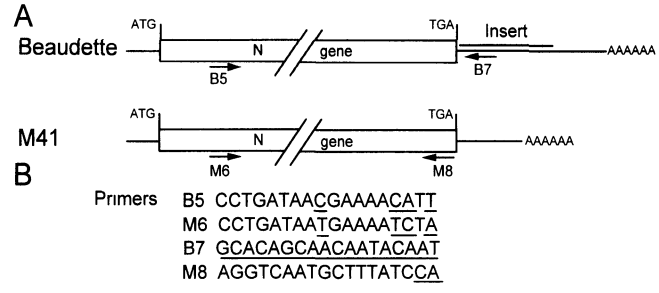

Figure 1. Strategy for detection of IBV-Beaudette-M41 recombinants (A) shows the position of the strain-specific primers in the two genomes (B) shows the oligonucleotide sequences with the mismatched nucleotides underlined

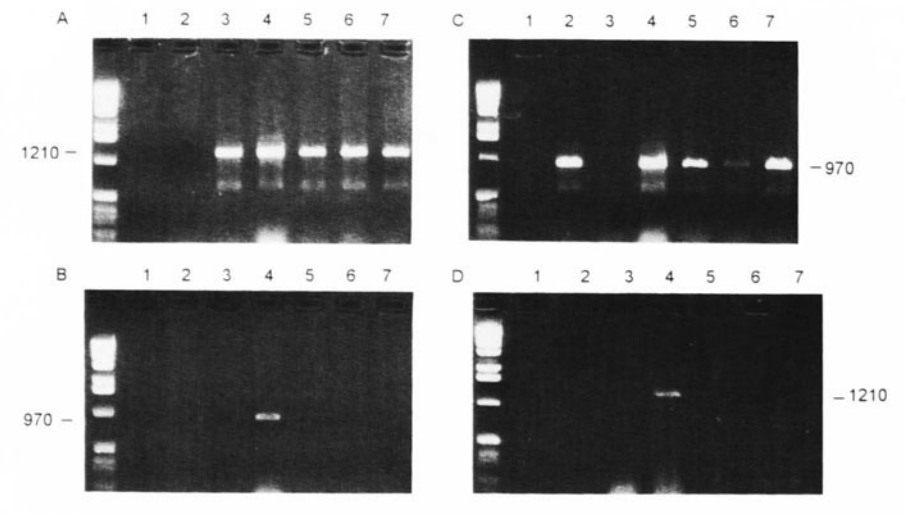

Figure 2. Detection of recombinants with strain-specific PCR, using different oligonucleotide primer combinations; (A) Beaudette specific B5+B7, (B) M41 specific M6+M8, (C) recombinant specific B5+M8, (D) recombinant specific M6+B7. For all primer combinations; lane 1, mock infected eggs; lane 2, M41-infected eggs; lane 3, Beaudette-infected eggs; lane 4-6 (respectively inoculations X1, X2, X3), M41- and Beaudette-coinfected eggs; lane 7, mixed M41 and Beaudette RNA. The marker used was the 1kb ladder from Boehringer.

detected with any primer combinations from the cDNA derived from eggs inoculated with PBSa, confirming that the PCR products were not derived from egg mRNAs. Furthermore, no PCR products were detected from cDNA derived from eggs infected with only IBV-M41 when the IBV-Beaudette specific primer combination was used (Fig. 2A, lane 2), whereas cDNA derived from eggs infected with Beaudette led to the expected PCR product of 1210 bp (Fig. 2A, lanes 3-7). In addition, no PCR products were detected from cDNA derived from eggs infected with only IBV-Beaudette when the IBV-M41 specific primer combination was used (Fig. 2B, lane 3), whereas cDNA derived from eggs infected with IBV-M41 led to the expected PCR product of 970 bp (Fig. 2B, lanes 2, 4-7), confirming that the primers were strain-specific. The primer combinations used to detect potential recombinants did not result in PCR products for cDNA derived from eggs infected with only IBV-M41 (Fig. 2C and 2D, lane 2) or infected only with IBV-Beaudette (Fig. 2C and 2D, lane 3) and did not give rise to any detectable products when cDNA derived from a mixture of Beaudette and M41 RNA was used (Fig. 2C and 2D, lane 7), indicating that no mispriming and no PCR artifacts had arisen under the chosen conditions. However, both recombinant primer combinations showed a product of the expected sizes (970 bp for primer combination B5+M8 and 1210 bp for primer combination M6+B7) when cDNA derived from coinfection X1 was used (Fig. 2C and 2D, lane 4), indicating that recombination had occurred between the two strains. These results were confirmed by three more independent experiments.

Although recombination was detected in one of the coinfections, recombination could not be detected in two other coinfections. The difference between these coinfections was the ratio of the amount of infectious Beaudette and M41 used to infect the eggs. The mixed infection in which recombination was detected had a ratio of Beaudette and M41 of approximally 1:5. However, if more Beaudette than M41 was used, recombination was not detected. This might be explained by the fact that IBV Beaudette has been adapted to grow in embryonated eggs and appears to grow much quicker in eggs than M41. These results

showed that it is important to use an appropriate ratio of viruses in a coinfection for detection of recombinants.

In order to confirm that the PCR products, derived from potential recombinants, consisted of both M41 and Beaudette sequences, they were cloned and eight clones were sequenced. Each recombinant contained sequences characteristic of both Beaudette and M41, showing that the DNA obtained had been derived from recombinants. Moreover, none of the recombinant DNAs had the same cross-over site, indicating that the PCR products were derived from several recombination events.

DISCUSSION

We have demonstrated experimentally that IBV strains undergo recombination during mixed infection. Although previous sequencing studies have provided circumstantial evidence that recombination occurs in IBV in the field (8,9,10), there was no direct experimental evidence that recombination did occur in IBV. This paper, however, describes the first experimental evidence of RNA-RNA recombination in IBV.

Strain-specific polymerase chain reactions were used for the detection of IBV recombinants because the technique is very sensitive and is extremely powerful for distinguishing between closely related strains. Further, it opens the possibility to study recombination in the absence of selection. All primers designed for the detection of IBV recombinants were shown to be able to differentiate between Beaudette and M41 and could therefore be used for detection of recombinants. Although oligonucleotide M8 had only two nucleotide mismatches in relation to Beaudette RNA, the primer was specific for M41. Previously, PCR had been shown to be strain-specific using an oligonucleotide with only 3 mismatches at the 3′ end (16).

To investigate the possibility of template-switching by the Taq-polymerase, RT-PCRs were conducted on a mixture of M41 and Beaudette RNA. PCR amplifications with primer combinations of one M41-specific primer and one Beaudette-specific primer did not result in a PCR product, indicating that template-switching of the Taq-polymerase was not detectable. This demonstrates that the observed PCR products from coinfected extracts did indeed arise from a recombination event by the coronavirus polymerase.

Sequencing of cloned PCR products confirmed that they were derived from IBV recombinants. All sequenced clones showed different recombination sites, suggesting that a number of recombination events had occurred. These recombination events seemed to occur randomly between the selected primers. Although initially it had been suggested that there might be favoured recombination sites in the coronavirus genome (7), it is now believed that the apparent clustering of recombination sites resulted from the use of selection pressure in the experiments (14,16). Banner *et al.*(14) demonstrated that recombination sites were randomly distributed after a mixed infection of two strains of MHV. However, when this mixed population was further passaged, recombination sites seemed to be clustered. To detect recombination in IBV, we used RT-PCR from pelleted virions from the mixed infections, without passaging to increases the chances of detecting recombination events, with as little selection pressure as possible, including recombinants with selective disadvantages.

Although PCR is a very powerful technique, it does have limitations. For example, the strain-specific PCR described in this paper can only detect recombinants that have an odd number of recombination events between the two selected primers. Further, recombination was detected in an area of less than 1/20th of the genome. Nevertheless, it is most likely that recombination also occurred in other parts of the genome, considering that recombination in MHV was detected in most areas of the genome.

The experimental evidence of recombination in IBV opens new possibilities for IBV research As in MHV, recombination between IBV genomic- and synthetic RNA could be examined, allowing the production of site-specific mutants For detection of this kind of recombination using synthetic RNA of the 3′ end of the genome, the same strain-specific primers can be used The possibility of creating various site-specific IBV recombinants by making use of the natural ability of IBV to recombine is an exciting prospect and might be useful for examining differences in pathogenicity among IBV strains

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