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Experimental Evidence of Recombination in Coronavirus Infectious Bronchitis Virus

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Embryonated eggs were coinfecting with two strains of the coronavirus avian infectious bronchitis virus (IBV), IBV-Beaudette and IBV-M41, to investigate whether recombination between the two strains would occur. Virions were isolated from the allantoic fluid of the coinfecting eggs and putative hybrid RNAs were detected by polymerase chain reaction (PCR), using strain-specific oligonucleotides. PCR products, of the expected sizes, were obtained as predicted from potential recombination events between the nucleoprotein (N) gene and the 3'-untranslated region of the two IBV genomes. Sequencing confirmed that they corresponded to hybrid RNAs. Virus produced as a result of the mixed infection was treated with an M41-specific neutralizing monoclonal antibody and passaged in Vero cells, in which IBV-Beaudette, but not IBV-M41, replicated. Hybrid RNA was still detectable after three serial passages. Since no IBV-M41 was detectable this confirmed that infectious recombinant genomes had been produced in the embryonated eggs. These findings not only support the circumstantial evidence, from sequencing studies of IBV field strains, that recombination occurs during replication of IBV and contributes to the diversity of IBV, but also show that coronavirus RNA recombination is not limited to mouse hepatitis virus. © 1995 Academic Press, Inc.

INTRODUCTION

Infectious bronchitis virus (IBV) is a member of the *Coronaviridae* (for reviews see Lai, 1990; Cavanagh *et al.*, 1994) and contains a single-stranded positive-sense RNA genome of 28 kb (Bournsnell *et al.*, 1987). It is a highly infectious and contagious pathogen of chickens which replicates mainly in the respiratory tract but also in some epithelial cells of the gut, kidney, and oviduct (King and Cavanagh, 1991; Lambrechts *et al.*, 1993). In order to study the basis of IBV pathogenesis, infectious cDNA clones allowing site-directed mutagenesis would be desirable. Infectious cDNA clones have been made for several RNA viruses (for review see Boyer and Heanni, 1994), but the large size of the IBV genome currently precludes their production. However, site-directed mutagenesis in the coronavirus mouse hepatitis virus (MHV) has recently been made possible using recombination (Koetzer *et al.*, 1992; van der Most *et al.*, 1992; Masters *et al.*, 1994).

Recombination in coronaviruses has been demonstrated experimentally using MHV (Lai *et al.*, 1985) and has been suggested to occur during both positive- and negative-strand RNA synthesis (Liao and Lai, 1992). A high frequency of recombination was found not only *in vitro* (Makino *et al.*, 1986) but also *in vivo* (Keck *et al.*, 1988). Recombination sites were detected over almost the entire genome and many recombinants were found with multiple crossover sites (as reviewed in Lai, 1992).

A recombination map, using temperature-sensitive MHV mutants, revealed the frequency of recombination to be approximately 1% per 1300 nucleotides. Assuming that recombination occurs randomly, the frequency of recombination in the entire genome was estimated to be around 25% (Baric *et al.*, 1990).

Although several sequencing studies on field isolates of IBV have provided circumstantial evidence that recombination had occurred in the field (Cavanagh and Davis, 1988; Kusters *et al.*, 1989, 1990; Cavanagh *et al.*, 1992; Wang *et al.*, 1993, 1994; Jia *et al.*, 1995), there has been no direct experimental evidence for recombination. To investigate recombination in IBV experimentally, we chose two strains of IBV, Beaudette and M41. Sequence analysis had indicated that both strains had a high sequence identity that may be expected to contribute to the production of recombinants as previously indicated by Kirkegaard and Baltimore (1986) for poliovirus. Comparison of the M41 nucleoprotein gene and the adjacent untranslated region (UTR) sequence with the corresponding region in Beaudette identified a 184-nucleotide deletion in M41 (Bournsnell *et al.*, 1985). The presence of this deletion permitted synthesis of a Beaudette-specific oligonucleotide. Synthesis of this and additional oligonucleotides, with differences in their 3' ends, made possible a polymerase chain reaction (PCR) method for the discrimination of recombinant and parental RNAs.

MATERIALS AND METHODS

Viruses and cells

IBV-M41 has been adapted to grow in chick kidney (CK) cells, whereas IBV Beaudette-US is a highly egg-

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adapted strain which, after passage in CK cells, has been passaged in Vero cells (Cavanagh *et al.*, 1986). Both strains were passaged once in 11-day-old specified pathogen-free Rhode Island Red (RIR) embryos for use in this work.

Vero cells were obtained from Flow Laboratories (ATCC, No. CCL81) and were grown in Eagle's MEM (Flow Laboratories) supplemented with 0.29% tryptose phosphate broth, 0.2% bovine serum albumin, 20 mM *N,N*-bis[2-hydroxyethyl]-2-aminoethanesulphonic acid, 0.2% NaHCO₃, 10 mM L-glutamine and antibiotics.

Coinfection of embryonated eggs with IBV M41 and Beaudette

Eleven-day-old RIR embryonated eggs were inoculated in quintuplicate in the allantoic cavity, with 7×10^6 egg infectious dose₅₀ (EID₅₀) IBV-Beaudette, or with 3×10^7 EID₅₀ IBV-M41, or coinfecting with 7×10^6 EID₅₀ IBV-Beaudette and 3×10^7 EID₅₀ IBV-M41 (X1), or with 7×10^6 EID₅₀ IBV-Beaudette and 3×10^6 EID₅₀ IBV-M41 (X2), or with 7×10^6 EID₅₀ IBV-Beaudette and 3×10^5 EID₅₀ M41 (X3), or inoculated with PBSa. Eggs were sealed with collodium and incubated at 37° for 24 hr. After overnight at 4° the allantoic fluid was harvested.

Extraction of RNA

Allantoic fluid from embryonated eggs infected with IBV was clarified by centrifugation in a Sorval RC-5B centrifuge with a Sorval SS-34 rotor for 30 min (700g) and the virions were pelleted by centrifugation in a Sorval ultracentrifuge using a Sorval AH629 rotor for 2 hr (30,000g). Virus RNA was extracted from the virions, or total cellular RNA from IBV-infected Vero cells, using the guanidinium isothiocyanate method of Chomczynski and Sacchi (1987) and dissolved in 50 μ l of nuclease-free water (Sigma) for virion extracts or 150 μ l for cell extracts.

Reverse transcription (RT)

cDNA syntheses were carried out using 3 μ l of either the virion or the total cellular RNA extracts following denaturation at 60° for 10 min in the presence of 0.21 nmol of oligonucleotide 100, complementary to the 3' end of the IBV-genome (Table 1). The mixture was immediately cooled on ice and incubated at 42° for 2 hr in a 35- μ l reaction mixture containing 250 U of M-MLV reverse transcriptase (GIBCO BRL), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 7 mM DTT, and 1.5 mM dNTPs.

Polymerase chain reaction

PCR amplifications were carried out using 5 μ l of the cDNA reactions in a total volume of 50 μ l containing 1.5 U *Taq* DNA polymerase (Promega), 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM MgCl₂, 0.2 mM dNTPs, and 0.3 nmol of positive-sense oligonucleo-

tides B7 or M8 and negative-sense oligonucleotides B5 or M6, respectively (Table 1). Reactions were performed at 94° for 1 min, 42° for 2 min, and 72° for 3 min for 25 cycles, with a final elongation step for 9 min at 72°, on a Hybaid Omnigene heating block. The positions of the oligonucleotides used for RT-PCR on the IBV genomes are shown in Fig. 1.

Cloning and sequencing

PCR products, derived from putative hybrid RNAs, were ligated into 50 ng pCR-II (Invitrogen TA cloning vector) 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.). The oligonucleotides M13n, M13p, 107, and 108 used for sequencing are shown in Table 1.

Neutralization of IBV M41 with MAb A38

Monoclonal antibody (MAb) A38 (Mockett *et al.*, 1984), produced against the spike (S) protein, was used to specifically neutralize IBV M41. Allantoic fluid or medium containing M41 at a titer of 1×10^6 pfu/ml was incubated with 0.2 vol of MAb A38 containing hybridoma culture fluid for 1 hr at room temperature. A 780-fold dilution of the MAb A38-containing hybridoma culture fluid completely neutralized 10^6 pfu/ml IBV M41.

Passage of IBV in Vero cells

Confluent cultures of Vero cells, in 25-cm² flasks, were washed three times with medium and inoculated with 0.5 ml undiluted allantoic fluid from embryonated eggs infected with either Beaudette or M41 or coinfecting with both IBV strains. After 1 hr incubation at 37° cells were washed three times in medium and incubated further at 37° in 5 ml medium for 16–40 hr until 50% of cells showed cytopathic effects (CPE). The medium was collected and used undiluted for further passage.

Hybridization of ³²P-labeled oligonucleotide to PCR products

Agarose gels, containing PCR products, were partially dried for 2 hr at 55° and directly hybridized with 4.2 nmol of ³²P-labeled oligonucleotide 104 (Table 1) for 5 hr at 37° in a Hybaid hybridization oven (Hybaid Ltd.) according to the method of Meinkoth and Wahl (1984). Oligonucleotide 104 was labeled using 1.5 MBq [γ -³²P]ATP (~111 TBq/mmol, NEG002A, DuPont) and 8 U T4 polynucleotide kinase (GIBCO-BRL) for 30 min at 37° and heated at 65° before use. Following hybridization, the gel was washed twice at 37° in wash buffer (0.18 M NaCl, 10 mM NaPO₄, pH 7.7, 1 mM EDTA, 0.2% SDS) and placed against X-ray film.

TABLE 1
Sequence and Application of the Oligonucleotides

Oligonucleotide	Sequence	Sense	Position ^a	Application
100	GCTCTAACTCTATACTAGCCT	–	27,588–27,608	RT
B5	CCTGATAACGAAAACATT	+	26,039–26,056	PCR
M6	CCTGATAATGAAAATCTA	+	26,039–26,056	PCR
B7	GCACAGCAACAATACAAT	–	27,233–27,250	PCR
M8	AGGTCAATGCTTTATCCA	–	26,990–27,007	PCR
M13n	TGACCGGCAGAAAATG	–	On plasmid	Sequencing
M13p	AACAGCTATGACCATG	+	On plasmid	Sequencing
107	TTGGCGCTGGAGAATTTCC	–	26,920–26,938	Sequencing
108	CCTTCCTCATTTCATCTTGTC	–	26,638–26,657	Sequencing
104	GCGTCTCCAGTATCCATG	–	26,074–26,092	Probe

^a Refers to the position of the nucleotides in the Beaudette genome (Boursnell *et al.*, 1987). The nucleotide positions for the M41-specific oligonucleotides M6 and M8 refer to the corresponding positions in the Beaudette sequence. All oligonucleotide sequences are in the 5' → 3' direction.

RESULTS

Design of strain-specific oligonucleotides

In order to detect putative hybrid IBV RNAs it was first necessary to design oligonucleotides that could distinguish between M41 and Beaudette using RT-PCR. For this, Beaudette and M41 sequences were analyzed for regions with the highest number of contiguous mismatches. Apart from the Beaudette insert, in the 3'-UTR, no differences were found of more than two consecutive nucleotides. Previously, Kwok *et al.* (1990) had shown that oligonucleotides with only two mismatches can be specific, provided that the mismatches are at the 3' end of the oligonucleotide. Figure 1 shows the oligonucleotides that were designed for use in PCR amplifications for the synthesis of products specific for either of the two IBV strains. Oligonucleotide B7 corresponds to a sequence found only in Beaudette; oligonucleotides B5 and M6 correspond to the same position in the two IBV

genomes but contain three of four nucleotides mismatching at their 3' ends. Oligonucleotide M8 has two adjacent nucleotide mismatches at the 3' end when compared to the same sequence in the Beaudette genome. The Beaudette-specific and M41-specific oligonucleotides were spaced 1210 and 970 nucleotides apart, respectively.

Embryonated eggs were infected with either M41 or Beaudette and virion RNA, extracted from virions isolated from the allantoic fluid, was used for the synthesis of cDNA using oligonucleotide 100, complementary to a sequence present within the 3'-UTR in both strains of IBV (Fig. 1). PCR amplifications were carried out on either the Beaudette- or the M41-derived cDNAs using either the Beaudette-specific oligonucleotides B5 and B7 or the M41-specific oligonucleotides M6 and M8. As can be seen from Fig. 2A, lane 3 (Beaudette cDNA using oligonucleotides B5 and B7) and Fig. 2B, lane 2 (M41 cDNA using oligonucleotides M6 and M8) the strain-specific oligonucleotides produced the expected 1210- and 970-bp PCR products, respectively. In contrast, the strain-specific oligonucleotides did not produce any product when used in conjunction with the heterologous strain (Fig. 2A, lane 2, M41 cDNA using oligonucleotides B5 and B7; Fig. 2B, lane 3, Beaudette cDNA using oligonucleotides M6 and M8). These results showed that the strain-specific oligonucleotide pairs were unable to generate DNA from the heterologous strain and were suitable for detecting hybrid RNAs derived from recombination events between the 3' ends of the genomes of the two IBV strains.

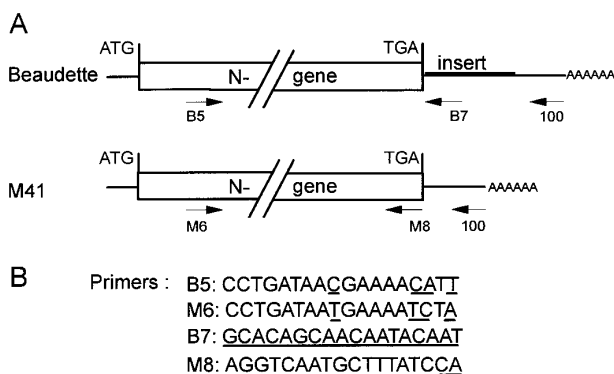


FIG. 1. Strategy for detection of IBV-Beaudette-M41 recombinants. (A) The position of oligonucleotide 100 used on the RNA of both strains for cDNA synthesis and the position of the strain-specific oligonucleotides, on the Beaudette and M41 genomes, used for PCR. (B) The sequences of the oligonucleotides used for PCR, with the mismatched nucleotides underlined.

Detection of IBV recombinants

To investigate whether recombination could occur in the 3' end of the IBV genome, embryonated eggs were coinfecting with the two strains of IBV, Beaudette and M41. IBV virions were isolated from the allantoic fluid

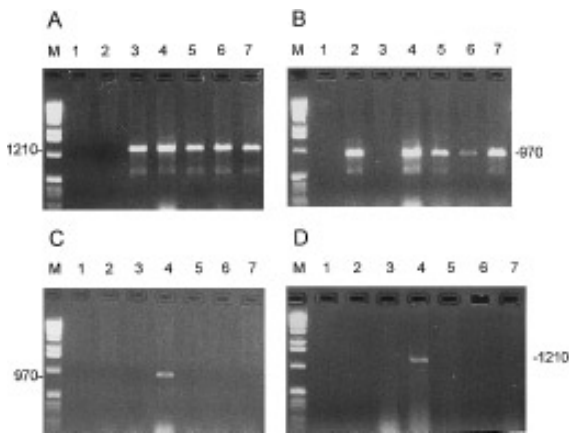


FIG. 2. Agarose gel analysis of the PCR products generated from oligonucleotide 100-derived cDNA. The different oligonucleotide combinations were: (A) Beaudette-specific B5 + B7; (B) M41-specific M6 + M8; (C) hybrid RNA-specific B5 + M8; (D) hybrid RNA-specific M6 + B7. For all oligonucleotide combinations: lane 1, RNA from mock-infected embryonated eggs; lane 2, virion RNA from M41-infected embryonated eggs; lane 3, virion RNA from Beaudette-infected embryonated eggs; lanes 4–6, virion RNA from Beaudette- and M41-coinfected embryonated eggs (with the following Beaudette:M41 ratios; lane 4, 1:5; lane 5, 2:1; and lane 6, 20:1); lane 7, simple mixture of M41 and Beaudette virion RNA. The fragments were electrophoresed in 1% agarose gels and the DNA was detected using ethidium bromide staining. The sizes of the Beaudette (1210 bp) and M41 (970 bp) PCR products are shown. The DNA size standard (M) used was the 1-kb ladder obtained from GIBCO BRL.

24 hr postinfection, RNA was extracted, and cDNA was synthesized from the RNA using oligonucleotide 100. Initial experiments indicated that excess oligonucleotide 100, from the RT reactions, resulted in PCR products of 1570 bp (Beaudette) and 1380 bp (M41), produced from oligonucleotides B5 or M6 and 100, respectively, in addition to the 1210-bp (Beaudette) and 970-bp (M41) PCR products derived from the strain-specific oligonucleotides. The presence of excess oligonucleotide 100 in PCRs containing mixed oligonucleotide pairs preferentially resulted in PCR products of 1570 or 1380 bp, produced from oligonucleotides 100 and B5 or M7, from the parental RNAs templates. No putative hybrid RNAs were detected using the mixed oligonucleotide pairs in the presence of oligonucleotide 100, presumably due to the low amounts of any putative hybrid RNA compared to the amounts of parental RNAs. Therefore, the amount of oligonucleotide 100 required in the RT reactions, for cDNA synthesis, which did not produce oligonucleotide 100-derived PCR products was determined prior to screening for putative hybrid RNAs (data not shown).

Reverse transcriptase and *Taq* polymerase have been shown to produce hybrid products due to template switching (Luo and Taylor, 1990; Meyerhans *et al.*, 1990). To check whether potential recombinant PCR products could result from RT-PCR artefacts, virion RNAs isolated from the allantoic fluid of embryonated eggs infected with only Beaudette or M41 were mixed and used as a control.

RT-PCR amplifications were also carried out on extracts from allantoic fluid from mock-infected embryonated eggs, to check whether any PCR products could have been produced from cellular RNA.

PCR amplifications were carried out, on oligonucleotide 100-derived cDNA samples, using the Beaudette-specific oligonucleotides B5 and B7 (Fig. 2A), the M41-specific oligonucleotides M6 and M8 (Fig. 2B), and the oligonucleotide combinations B5 and M8 (Fig. 2C) and M6 and B7 (Fig. 2D) for the detection of putative hybrid RNAs. The Beaudette-specific oligonucleotides produced the 1210-bp Beaudette-derived PCR product from embryonated eggs coinfecting with both IBV strains (Fig. 2A, lanes 4–6). Similarly, the M41-specific oligonucleotides produced the 970-bp M41-derived PCR product from embryonated eggs coinfecting with both IBV strains (Fig. 2B, lanes 4–6). The Beaudette- and M41-specific oligonucleotides produced the 1210- and 970-bp PCR products, respectively, from cDNA synthesized from the control mixture of RNA, prepared by mixing RNA extracted from embryonated eggs infected with one strain or the other (Figs. 2A and 2B, lane 7). These observations showed that the strain-specific oligonucleotide pairs only detected the appropriate parental virus RNA from virion-derived RNAs isolated from either coinfecting embryonated eggs or control mixtures of RNA.

Mixed oligonucleotide pair combinations, B5 + M8 or M6 + B7, used for the detection of putative hybrid RNAs, did not result in PCR products from cDNAs synthesized from virion RNA from embryonated eggs infected with only M41 (Figs. 2C and 2D, lane 2), Beaudette (Figs. 2C and 2D, lane 3), or with the control mixture of both virion RNAs (Figs. 2C and 2D, lane 7). These results indicated that mispriming of the strain-specific oligonucleotides did not occur and that no RT-PCR artefact products were produced as a result of template switching between the two RNA or cDNA templates.

Mixed oligonucleotide combinations produced RT-PCR products of the expected sizes, 970 bp for oligonucleotide combination B5 + M8 and 1210 bp for oligonucleotide combination M6 + B7, from coinfection X1 (Figs. 2C and 2D, lane 4). These results indicated that virions isolated from the allantoic fluid of coinfecting embryonated eggs contained hybrid RNAs. No RT-PCR products were detected from allantoic fluid extracts produced from mock-infected embryonated eggs using either the strain-specific oligonucleotide pairs or the mixed oligonucleotide combinations. This confirmed that the RT-PCR products observed above were not derived from any cellular RNAs present in the allantoic fluid. All results were confirmed by three independent experiments.

Although putative hybrid RNAs were detected in one of the coinfections, X1, they were not detected in the two other coinfections, X2 and X3. The coinfections differed in the ratios of the amounts of Beaudette and M41 virus used to infect the embryonated eggs. The coinfection in

which potential recombinant RNAs were detected, X1, had a ratio of Beaudette:M41 of approximately 1:5 EID₅₀. We consistently observed that if more Beaudette than M41 was used (i.e., ratios of Beaudette:M41 of ~2:1 (X2) or ~20:1 (X3)) putative hybrid RNAs were not detected by the above method. This observation might result from the fact that IBV Beaudette has been adapted to grow in embryonated eggs and appears to grow much faster in embryonated eggs than M41. The combination of more Beaudette than M41 and the previous observation that Beaudette grew quicker in embryonated eggs than M41 might result in Beaudette preferentially infecting more cells. Alternatively, the higher amount of Beaudette might either preclude M41 from efficiently infecting the eggs or that it outgrew any potential recombinant virus to a point that such putative hybrid RNAs could not be detected by strain-specific PCR. These results indicated that it was important to use an appropriate ratio of viruses in a coinfection for detection of recombinants. It should be noted that the ratio of the control mixed RNAs, derived from virion RNA isolated from embryonated eggs infected with only one or the other strain of IBV, were as close as possible to the ratio of input viruses used for the X1 coinfection.

Overall, these observations supported the proposal that the RT-PCR products had been derived from hybrid RNAs resulting from recombination events between the two IBV strains during mixed infection.

Cloning and sequencing of PCR products derived from recombinant RNA

To verify that the PCR products, derived from hybrid RNAs using either oligonucleotide pairs B5 + M8 or M6 + B7, consisted of both M41 and Beaudette sequences, they were cloned and sequenced. Eight clones were examined, four from each of the oligonucleotide combinations, containing PCR products of 1210 bp (clones C1 to C4) and 970 bp (clones D1 to D4), respectively.

Figure 3 shows the alignment of the eight sequences, C1 to C4 and D1 to D4, derived from the putative hybrid RNAs together with the published sequences of the corresponding genome regions from Beaudette and M41 (Bournsnel *et al.*, 1985). Each of the PCR product sequences contained nucleotides characteristic of both the Beaudette and the M41 sequences, indicating that the PCR products were derived from hybrid RNAs resulting from recombination events between the two IBV strains. As can be seen from Fig. 3, Beaudette and M41 have only a few nucleotide differences within the genome region being examined; therefore, the exact crossover sites of the hybrid RNAs could not be determined. The crossover sites between the two parental IBV sequences, shown as boxes in Fig. 3, are defined as lying within regions where the sequence characteristic for one strain changed to the sequence characteristic of the other

strain. For example, clone D1 had the first 161 nucleotides characteristic of M41 with a potential crossover region of 26 nucleotides and thereafter the nucleotides were characteristic of Beaudette. None of the eight sequences had the same crossover site, indicating that the RT-PCR products were derived from hybrid RNAs resulting from several recombination events. It should be noted that at this stage we could not say whether any of these hybrid RNAs resulted in recombination events leading to infectious genomic RNA.

Although the eight sequences consisted of both M41- and Beaudette-like sequences, some nucleotide differences were observed between the M41 and Beaudette sequences present in the PCR products, as shown in Fig. 3, and those in the published sequences (Bournsnel *et al.*, 1985). This could partly have been due to RT-PCR errors. However, in some cases the published M41 sequence differed from the sequences determined for several of the hybrid RNAs, as illustrated in Fig. 3 by arrows. These observations indicated a change in the parental M41 sequence, of the particular M41 strain used in this work, from that of the published M41 sequence. The nucleotide differences observed between the previously published sequence of M41 and the sequence of the M41 virus used in these experiments might have resulted from adaptation of the M41 to CK cells.

Passage of IBV in Vero cells following neutralization of IBV-M41 with MAb A38

To determine whether any of the hybrid RNAs were genomic RNA that could replicate, we passaged virus isolated from the allantoic fluid of coinfecting embryonated eggs, shown to contain virion-derived hybrid RNAs. To reduce the possibility of the two parental viruses outgrowing recombinant virus and to further reduce the possibility that the hybrid RNA was a result of some RT-PCR artefact due to the presence of the two parental genomes, we decided to remove one of the two parental strains.

One potential method comprised the passage of the viruses in a cell line, Vero, previously demonstrated to sustain the growth of Beaudette but not M41. The Vero cells should therefore act as a selective cell allowing the growth of Beaudette but not M41 from a mixed population. Previous studies (unpublished data) had shown that no CPE was observed following the infection of Vero cells with M41. However, we did not know if IBV RNA was produced in M41-infected Vero cells or the effect of M41 in Vero cells in the presence of Beaudette. Allantoic fluid either containing only one parental strain, Beaudette or M41, or containing potential recombinants in the presence of both parental viruses was passaged in Vero cells (P₁) followed by passaging progeny virus an additional two times in Vero cells (P₂ and P₃). RNA was extracted from each passage, cDNA was synthesized using oligo-

	26039															
Beaud	TTCCGTGATAACGAAAACATTAAGCCAGCCAGCA ACATGGATAC TGGAGACGCCAAGCCAGGTTTAAGCCAGGCCAAAGGTGGAAGAAAACCCAGTCCCAGATGCTTGGTAC															
C1	C	CA	T	G	C	A	C	C	C	C	C	C	C	C	C	C
C2	C	CA	T	G	C	A	T	T	T	T	T	T	T	T	T	T
C3	C	CA	T	G	C	A	C	C	C	C	C	C	C	C	C	C
C4	C	CA	T	G	C	A	C	C	C	C	C	C	C	C	C	C
D1	T	TC	A	A	T	G	T	T	T	T	T	T	T	T	T	T
D2	T	TC	A	A	T	G	T	T	T	T	T	T	T	T	T	T
D3	T	TC	A	A	T	G	T	T	T	T	T	T	T	T	T	T
D4	T	TC	A	A	T	G	T	T	T	T	T	T	T	T	T	T
M41	TTCCGTGATAATGAAAATCTAAAGTCAAGCCAGCA GCATGGATAC TGGAGACGCCAAGCTACGTTTAAGCCAGGCCAAAGGTGGAAGAAAACCCAGTCCCAGATGCTTGGTAT															
	26149															
Beaud	TTTTACTATACTGGAACAGGACCTGCCGTGACCTGAACTGGGGTGATAC TCAAGATGGTATAGTGTGGGTGCTGCG TAAGGGTGC TGATACTAAATCTAGATCCAATCA															
C1	T	G	A	C	T	C	T	C	T	C	T	C	T	C	T	C
C2	A	G	A	C	T	G	C	T	G	C	T	G	C	T	G	C
C3	T	G	A	C	T	G	C	T	G	C	T	G	C	T	G	C
C4	T	G	A	C	T	G	C	T	G	C	T	G	C	T	G	C
D1	A	A	A	T	G	C	T	G	C	T	G	C	T	G	C	T
D2	A	A	A	T	G	C	T	G	C	T	G	C	T	G	C	T
D3	A	A	A	T	G	C	T	G	C	T	G	C	T	G	C	T
D4	A	A	A	T	G	C	T	G	C	T	G	C	T	G	C	T
M41	TTTTACTATACTGGAACAGGACCTGCCGTGAACTGAACTGGGGTGATAC CCAAGATGGTATAGTGTGGGTGCTGCG TAAGGGTGC TGATACTAAATCTAGATCCAATCA															
	26259															
Beaud	GGGTACAAGAGATCCTGATAAAGTTTGACCAATACCCACTACG ATTC TCGGATGG CGGACCTGATGGTAATTTCCGTTGGGAC TTCATTCCCTTGAAACCGTGGTAGGAGTG															
C1	AA	A	TC	T	C	A	G	T	A	C	A	T	C	A	T	C
C2	TC	T	CT	C	T	G	T	A	G	T	A	C	A	T	C	C
C3	AA	A	TC	T	C	A	G	T	A	C	A	T	C	A	T	C
C4	AA	A	TC	T	G	C	A	A	C	G	T	N	C	C	C	T
D1	AA	A	TC	T	C	A	A	A	C	G	T	C	C	C	C	T
D2	TC	T	CT	C	T	G	G	G	CT	A	C	A	C	T	T	C
D3	TC	T	CT	C	T	G	G	G	T	A	C	A	C	T	T	C
D4	TC	T	CT	C	T	G	G	T	A	C	A	C	T	C	C	T
M41	GGGTACTCGTGACTCTGACAAGTTTGACCAATATCCGCTACG ATTT TCAGACGG AGGACCTGATGGTAATTTCCGTTGGGAT TTCATTCCCTGGAATGGTGGTAGGAGTG															
	26369															
Beaud	G AAGATCAACAGCAGCTTCATCAGCAGCAG CTAGTAGAGCACCATCAGTGAAGGTTTCGCGTGGTGGTTCGTAAGGTGATTCGGAAGATGACCTTATTGCTCGTGACGCAAAAG															
C1	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
C2	G	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
C3	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
C4	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
D1	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
D2	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
D3	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
D4	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
M41	G AAGATCAACAGCAGCTTCATCAGCAGCAG CTAGTAGAGCACCATCAGTGAAGGTTTCGCGTGGTGGTTCGTAAGGTGATTCGGAAGATGACCTTATTGCTCGTGACGCAAAAG															
	26479															
Beaud	ATAATCCAGGATCAGCAGAAAAAGGGCTCTGCATTACCAAGGCCAAGGCAGATGAAATGGCTACGCCGGTATTGCAAGCGCACTATCCACCTAATTATA GGGTTGA															
C1	T	G	T	T	A	T	T	T	T	T	T	T	T	T	T	T
C2	T	G	T	T	A	T	T	T	T	T	T	T	T	T	T	T
C3	T	G	T	T	A	T	T	T	T	T	T	T	T	T	T	T
C4	T	G	T	T	A	T	T	T	T	T	T	T	T	T	T	T
D1	C	A	C	T	C	A	A	A	T	C	C	T	T	T	N	A
D2	T	G	T	T	A	T	T	T	T	C	C	T	T	T	T	A
D3	T	G	T	T	A	T	T	T	T	C	C	T	T	T	T	A
D4	C	A	C	T	C	A	A	A	T	C	C	T	T	T	T	A
M41	ATAATCCAGGATCAGCAGAAAAAGGGTCTCTGCATTACCAAGGCCAAGGCAGATGAAATGGCTACGCCGGTATTGCAAGCGCACTATCCACCTAATTATA GGGTTGA															
	26589															
BEAUD	TCAAGTGTGGTCCCCTACTAAAGGTAAGGAGGGG AATTTTGGTGATGACAAGATGAATGAGGAAGGTATTAAGGATGGCG TGTTACAGCAATGCTCAACCTAGTC C															
C1	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
C2	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
C3	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
C4	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
D1	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
D2	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
D3	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
D4	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
M41	TCAAGTGTGGTCCCCTACTAAAGGTAAGGAGGGG AATTTTGGTGATGACAAGATGAATGAGGAAGGTATTAAGGATGGCG CGTTACAGCAATGCTCAACCTAGTT C															
	26699															
Beaud	CTAGCAGCCATGCTTGCTTTTGGAAGTAGAGTGACACCCAAACTTCAACTAGATGGCTTCACTTGGAGATTTGAATTTACTACTGTGGTCCCATGTGATGACCCGACG															
C1	C	G	G	G	C	G	G	G	A	A	A	C	T	T	C	
C2	C	G	G	G	C	G	G	G	A	A	A	C	T	T	C	
C3	C	G	G	G	C	G	G	G	A	A	A	C	T	T	C	
C4	C	G	G	G	C	G	G	G	A	A	A	C	T	T	C	
D1	T	T	T	T	A	A	T	T	T	T	T	T	T	T	C	N
D2	T	T	T	T	A	A	T	T	T	T	T	T	T	T	T	C
D3	T	T	T	T	A	A	T	T	T	T	T	T	T	T	T	C
D4	T	T	T	T	A	A	T	T	T	T	T	T	T	T	T	C
M41	CTAGCAGCCATGCTTGCTTTTGGAAGTAGAGTGACGCCCCAAACTTCAACCAGATGGCTGACCTTGAATTTGAATTTACTACTGTGGTCCCACGTGATGATCCGACG															
	26809															
BEAUD	TTTGATAATTATGTGAAAATTTGTGATCAGTGTGTGATGGTGTAGGAACGCGTCCAAAAGATGACGAACCAA AACCAAAAGTCACGCTCAAGTTCAAGACCTGCT ACAAG															
C1	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
C2	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
C3	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
C4	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T
D1	G	C	G	C	G	N	C	AN	TG	TG	TG	TG	TG	TG	TG	TG
D2	G	C	G	C	G	N	C	AN	TG	TG	TG	TG	TG	TG	TG	TG
D3	G	C	G	C	G	N	C	AN	TG	TG	TG	TG	TG	TG	TG	TG
D4	G	C	G	C	G	N	C	AN	TG	TG	TG	TG	TG	TG	TG	TG
M41	TTTGATAATTATGTGAAAATTTGTGATCAGTGTGTGATGGTGTAGGAACGCGTCCAAAAGATGATGAACCAA GACCAAAGTCACGCTCAAGTTCAAGACCTGCA ACAAG															
	26919															
BEAUD	AGGAAATTCCTCCAGCGCCAAAGACAAGCAGCGCCAAAGAAAGGAGAAAAGCTAAAGAAAGCAGGATGATGAAGCAGATAAAGCATTGACCT															
C1	G	T	T	T	G	C	T	TG	TG	TG	TG	TG	TG	TG	TG	TG
C2	G	T	T	T	G	C	T	TG	TG	TG	TG	TG	TG	TG	TG	TG
C3	G	T	T	T	G	C	T	TG	TG	TG	TG	TG	TG	TG	TG	TG
C4	G	T	T	T	G	C	T	TG	TG	TG	TG	TG	TG	TG	TG	TG
D1	A	A	A	A	T	N	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA
D2	A	A	A	A	T	N	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA
D3	A	A	A	A	T	N	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA
D4	A	A	A	A	T	N	CA	CA	CA	CA	CA	CA	CA	CA	CA	CA
M41	AGGAAATTCCTCCAGCGCCAAAGACAAGCAGCGCCAAAGAAAGGAGAAAAGCTAAAGAAAGCAGGATGATGAAGCAGATAAAGCATTGACCT															

nucleotide 100, and the strain-specific oligonucleotide pairs B5 + B7 or M6 + M8 were used to detect either Beaudette- or M41-derived cDNA by PCR.

The RT-PCR products obtained from RNA extracts of passages P₁, P₂, and P₃ are shown in Fig. 4. The Beaudette-specific oligonucleotide combination B5 + B7 identified the expected 1210-bp PCR product in all three passages from allantoic fluid-containing Beaudette (Fig. 4A, lanes 2, 3, 5, 6, 8, and 9) but not from allantoic fluid isolated from eggs infected with only M41 (Fig. 4A, lanes 1, 4, and 7). These results indicated that Beaudette was capable of growing in Vero cells in the presence of M41. The M41-specific oligonucleotide combination M6 + M8 detected the 970-bp PCR product in Vero cells, infected with allantoic fluid from embryonated eggs infected only with M41, only in P₁ (Fig. 4B, lane 1). No M41-derived PCR products were detected in the subsequent passages P₂ and P₃ in Vero cells (Fig. 4B, lanes 4 and 7). No M41-derived PCR products were detected in Vero cells only infected with Beaudette (Fig. 4B, lanes 2, 5, and 8). These observations indicated that although the M41 present in allantoic fluid was initially able to infect Vero cells it subsequently failed to grow. This indicated that either no viable virus was produced in Vero cells at P₁ or that virus, if produced, was not released or that too little virus was released to successfully initiate infection. However, analysis of RNA extracts from Vero cells infected with allantoic fluid isolated from coinfecting embryonated eggs using the M41-specific oligonucleotide pair identified the 970-bp M41-derived PCR product in all three passages (Fig. 4B, lanes 3, 6, and 9). This result indicated either that the Beaudette virus had been able to rescue the M41 genome for subsequent passage through Vero cells or that the M41 PCR product had been derived from hybrid Beaudette viruses containing M41 sequences with the crossover sites upstream of the oligonucleotide B5/M6 sites. As it was not possible to differentiate between these two possibilities, a different approach to remove M41 was examined.

A second method for removing one virus in a mixed population is by the use of specific neutralizing MAbs. For such a purpose we used one such MAb, A38, which has been demonstrated to selectively neutralize M41 but not Beaudette (Mockett *et al.*, 1984). To determine the amount of MAb A38 required various dilutions of a MAb A38-containing hybridoma, culture fluid was used to neutralize M41. We determined that a 780-fold dilution of the

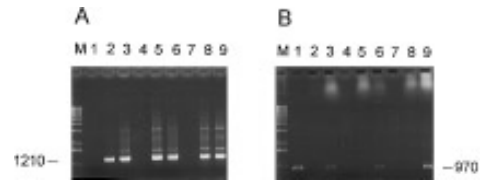


FIG. 4. PCR analysis of Vero cell extracts following infection with virus present in the allantoic fluid of embryonated eggs previously infected with M41 or Beaudette or coinfecting with both viruses. Progeny virus were passaged three times in Vero cells, RNA was extracted, and cDNA was generated using oligonucleotide 100. PCRs were carried out on the cDNAs, using (A) Beaudette- and (B) M41-specific oligonucleotides, B5 + B7 or M6 + M8, respectively, generated from Vero cells infected with M41 (lanes 1, 4, and 7) or Beaudette (lanes 2, 5, and 8) or coinfecting with M41 and Beaudette (lanes 3, 6, and 9). The three passages were: lanes 1 to 3, P₁; lanes 4 to 6, P₂; lanes 7 to 9, P₃. The fragments were electrophoresed in 1% agarose gels and the DNA was detected using ethidium bromide staining. The sizes of the Beaudette (1210 bp) and M41 (970 bp) PCR products are shown. The DNA size standards (M) used was the 1 kb ladder obtained from GIBCO BRL.

hybridoma culture fluid completely neutralized 10⁶ pfu/ml of M41. The titers of M41 in allantoic fluid obtained from M41-infected embryonated eggs were observed to be no more than 10⁶ pfu/ml. A 20-fold dilution of the A38 hybridoma culture medium was subsequently used to neutralize the M41 in either allantoic fluid from coinfecting eggs or potentially in the medium from Vero cells. This dilution of MAb A38 was shown to have no effect on Beaudette.

Ideally at least two such antibodies should be used in order to eliminate the possibility of selecting escape mutants. However, because we only had one MAb capable of neutralizing M41 but not Beaudette, we decided to examine the elimination of M41 virus from a mixed population using a combination of virus neutralization with MAb A38 followed by passage in Vero cells. Results of the experiments described above indicated that no M41 was detectable, following neutralization of M41 in allantoic fluid, in RNA extracts following subsequent passage on Vero cells. This indicated that if any MAb A38-resistant M41 viruses were selected from the allantoic fluid they would be present in such low amounts that it would be unlikely there would be subsequent rescue of M41 genomes by Beaudette. We proposed that neutralization of M41 with A38 followed by subsequent passage on Vero cells would eliminate M41. Therefore, the detection of hybrid RNAs, following neutralization of M41 with

FIG. 3. Alignment of the DNA sequences of the PCR products derived from the hybrid RNAs. The corresponding regions of Beaudette (top) and M41 (bottom) are included (from Bournsnel *et al.*, 1985). Sequences C1 to C4 and D1 to D4 were produced using oligonucleotides B5 + M8 and M6 + B7, respectively. The nucleotides of the PCR product sequences are only presented where differences were observed between the aligned sequences. Crossover sites are indicated as boxed areas and are defined as those regions in which the sequence of the hybrid RNA changes from one parental sequence to the other parental sequence. Areas that were not sequenced are indicated with dots; positions where the nucleotides were not certain are indicated by an N. Nucleotides that were identified as different between the PCR products and the published M41 sequence (Bournsnel *et al.*, 1985) are indicated with an arrow. The numbers above the sequences refer to the nucleotide positions for the complete sequence of the Beaudette genome.

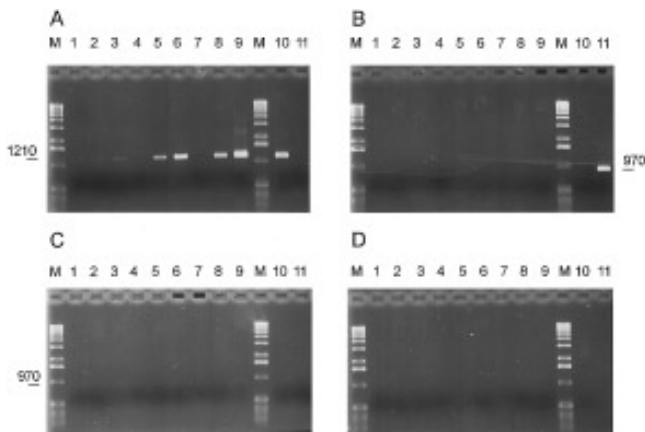


FIG. 5. PCR analysis of Vero cell extracts following infection with virus, pretreated with MAb A38, from the allantoic fluid of embryonated eggs previously infected with M41 or Beaudette or coinfecting with both viruses. Progeny virus was treated with MAb A38 and passaged three times on Vero cells. RNA was extracted and cDNA was generated using oligonucleotide 100. PCRs were carried out on the cDNAs using (A) Beaudette- and (B) M41-specific oligonucleotides, B5 + B7 or M6 + M8, respectively, or combinations of the oligonucleotides (C) B5 + M8 and (D) M6 + B7 to detect hybrid RNA. The samples used to infect the Vero cells were M41 (lanes 1, 4, and 7) or Beaudette (lanes 2, 5, and 8) or they were coinfecting with M41 and Beaudette (lanes 3, 6, and 9). The three passages were: lanes 1 to 3, P₁; lanes 4 to 6, P₂; lanes 7 to 9, P₃. The fragments were electrophoresed in 1% agarose gels and the DNA was detected using ethidium bromide staining. The sizes of the Beaudette (1210 bp) and M41 (970 bp) PCR products are shown. The DNA size standard (M) used was the 1-kb ladder obtained from GIBCO BRL. PCRs were carried out on control cDNA derived from Beaudette (lane 10) and M41 (lane 11) RNA.

MAb A38 followed by selective passage of resulting virus on Vero cells, would indicate that recombinant viruses existed in the presence of Beaudette and could not arise as a result of some RT-PCR artefact due to the presence of the two virus genomes.

Samples of allantoic fluid, previously used for the analysis of growth of M41 in Vero cells, were treated with MAb A38 to neutralize M41. The A38-treated allantoic fluid samples were used to infect Vero cells (P₁) and progeny virus subsequently passaged, following treatment with MAb A38, twice in Vero cells (P₂ and P₃). RNA was extracted from the P₁ to P₃ Vero cells, cDNA was synthesized using oligonucleotide 100, and the presence of parental- or hybrid-derived cDNAs was analyzed using the strain-specific oligonucleotides.

The Beaudette-specific oligonucleotide combination B5 + B7 produced Beaudette-derived PCR products in all three Vero cell passages, following infection with allantoic fluid derived from embryonated eggs infected with either Beaudette (Fig. 5A, lanes 2, 5, and 8) or Beaudette and M41 (Fig. 5A, lanes 3, 6, and 9). No Beaudette-derived PCR products were detected in Vero cell extracts following infection with allantoic fluid from eggs infected with only M41 (Fig. 5A, lanes 1, 4, and 7). The M41-specific oligonucleotide combination M6 + M8 did not

produce any M41-derived PCR products in any Vero cell extract following infection with any of the allantoic fluid samples (Fig. 5B). To discount the possibility that some PCR products may have been produced from the Vero cell extracts but were not detectable under the conditions used, the gels were hybridized with radiolabeled oligonucleotide 104 (Table 1). No M41-derived PCR products were detected (data not shown). These results confirmed that the combination of the neutralizing MAb A38 followed by selective passage on Vero cells eliminated M41 even in the presence of Beaudette. These observations supported our previous results that PCR-derived products derived from hybrid RNAs were not a result of RT-PCR artefacts due to the presence of the two virus genomes, because one parental virus had been eliminated from the samples.

The oligonucleotide combination B5 + M8 did not produce any hybrid RNA-derived RT-PCR products in any of the three Vero cell passages infected with allantoic fluid samples from eggs infected with either Beaudette (Fig. 5C, lanes 2, 5, and 8) or M41 (Fig. 5C, lanes 1, 4, and 7). However, this oligonucleotide combination did produce a 970-bp PCR product from Vero cell extract P₃ from cells infected with allantoic fluid derived from eggs coinfecting with both IBV strains (Fig. 5C, lane 9). This result indicated that a potential hybrid RNA had been passaged from the allantoic fluid of coinfecting eggs, previously shown to contain hybrid RNAs, in the absence of one of the parental strains, M41. The oligonucleotide combination M6 + B7 did not produce any hybrid-derived PCR products in any Vero cell passage using any of the allantoic fluid samples (Fig. 5D). This indicated that no hybrid RNAs were detected as a result of potential recombinants consisting of predominantly M41 sequence with Beaudette sequence at the 3' end. Potential viruses of this nature would be presumably be like the parental M41 strain and have been eliminated by passage in Vero cells following neutralization with MAb A38.

Cloning and sequencing of the PCR products from potential recombinants detected after passage in Vero cells

The 970-bp PCR product derived from the putative hybrid RNA detected in Vero cell P₃, following selective passage of virions from the allantoic fluid of embryonated eggs coinfecting with the two IBV strains, was cloned and partially sequenced. Analysis of the 5' end of the PCR product from four clones (VPCR1 to VPCR4) showed it to contain M41-specific nucleotides preceded by a short region of Beaudette-specific sequence (Fig. 6A). The G residue at position 26,061 on the PCR product was derived from the Beaudette sequence, although the published sequence for M41

A

26039

Beaud * ** * * * * *

TTCTGTATAACGAAAAACATTAAGCCAAGC CAGCA ACATGGATACTGGAGACGCCAAGCCAGGTTTAAGCCAGGCAAAAGGTGGAAGAAAAACCAGTCCCAGATGCTTGGTAT

VPCR1 CAGCA GCATGGNTATTGGAGACGCCAAGCTAGGTTTANGCCAGGTAAGGTGGAAGAAAAACCAGTCCCAGATGCTTGGTAT

VPCR2 CAGCA GCATGGATATTGGAGACGCCAAGCTAGGTTTAAAGCCAGGTAAGGTGGAAGAAAAACCAGTCCCAGATGCTTGGTAT

VPCR3 CAGCA GCATGGATATTGGAGACGCCAAGCTAGGTTTANGCCAGGTAAGGTGGAAGAAAAACCAGTCCCAGATGCTTGGTAT

VPCR4 CAGCA GCATGGATATTGGAGACGCCAAGCTAGGTTTAAAGCCAGGTAAGGTGGAAGAAAAACCAGTCCCAGATGCTTGGTAT

M41 - CK

M41 AC T G T

TTCTGTATAATGAAAACTAAAGTCAAGC CAGCA GCATGGATACTGGAGACGCCAAGCTACGTTTAAGCCAGGCAAAAGGTGGAAGAAAAACCAGTCCCAGATGCTTGGTAT

26149

Beaud * * * * * *

TTTTACTATACTGGAACAGGACCTGCCGCTGACCTGAAC TGGGGTGATAC TCAAGATGGTATAGTGTGGGTTGCTGCTAAGGGTGC T GATACTAAATCTAGATCCAATCA

VPCR1 TCC TACTATACTGGAACAGGACCGCCGCTNACCTGAATTGGGGTGATAGCCAAGATGGTATAGTGTGGGTTGCTGGT - - - - -

VPCR2 TTTTACTATACTGGAACAGGACCGCCGCTAACTGAATTGGGGTGATAGCCAAGATGGTATAGTGTGGGTTGCTGGT AAGGGTGC T GATACTAAATTTAGATCTAATCA

VPCR3 TTCTACTATACT

VPCR4 TTCTACTATACTGGAACAGGACCGCTGCC TAACTGAATTGGGGTGATAGCCAAGATGGTATAGTGTGGGTTGCTGGT AAGGGTGC T GATACTAAATTTAGATCTAATCA

M41 - CK

M41 A

TTTTACTATACTGGAACAGGACCTGCCGCTAACTGAATTGGGGTGATAGCCAAGATGGTATAGTGTGGGTTGCTGGT AAGGGTGC T GATACTAAATTTAGATCTAATCA

B

26809

BEAUD * * * * * *

TTTGATAATTATGTAAAAATTTGTGATCAGTGTGTTGATGGTGTAGGAACCGCTCCAAAAGATGATGAACCAAACAAAGTCAAGTTC AAGACCTGC TACAAG

VPCR1 TTTGATAANTATGCAAAAACNTGTGATCAGTGTCTTGATGGTGGAGGAACNCGCTCCAACAGATGATGAACCGAGACCANAGCCACGNTC NAGGTC TAGACCTNCAGCAAG

VPCR2 TTTGATAATTATGTANAAATTTGTGATCAGTGTGTTGATGGTGTAGGAACCGCTCCNAAAGATGATGAACCAAACAAAGTCAAGTTC AAGACCTGC AACAAG

VPCR3 TTTGATAATTATGTAAAAATTTGTGATCAGTGTGTTGNTGGTGTAGGAACCGCTCCAAAAGATGATGAACCAAACAAAGTCAAGTTC AAGACCTGC AACAAG

VPCR4 TTTGATAATTATGTAAAAATTTGTGATCAGTGTGTTGATGGTGTAGGAACCGCTCCAAAAGATGATGAACCAAACAAAGTCAAGTTC AAGACCTGC AACAAG

M41 - CK

M41

TTTGATAATTATGTAAAAATTTGTGATCAGTGTGTTGATGGTGTAGGAACCGCTCCAAAAGATGATGAACCAAACAAAGTCAAGTTC AAGACCTGC AACAAG

26919

BEAUD * * * * * *

AGGAAATTC TCCAGCGCCAAGACAACAGCGCCCAAAGAAAGGAGAAAAAGCTAAAGAAAGCAGGATGATGAAGCAGATAAAGCATTGACCT

VPCR1 AGGAAATTC TCCAGCGCCAAGACAACAGCGCCCAAAGAAAGGAGAAAAAGCTAAAGAAAGCAGGATGATGAAGCAGATAAAGCATTGACCT

VPCR2 AGGAAATTC TCCAGCGCCAAGACAAGCAGCGCCCAAAGAAAGGAGAAAAAGCTAAAGAAAGCAGGATGATGAAGTGGATAAAGCATTGACCT

VPCR3 AGGAAATTC TCCAGCGCCAAGACAAGCAGCGCCCAAAGAAAGGAGAAAAAGCTAAAGAAAGCAGGATGATGAAGTGGATAAAGCATTGACCT

VPCR4 AGGAAATTC TCCAGCGCCAAGACAAGCAGCGCCCAAAGAAAGGAGAAAAAGCTAAAGAAAGCAGGATGATGAAGTGGATAAAGCATTGACCT

M41 - CK

M41 G

AGGAAATTC TCCAGCGCCAAGACAAGCAGCGCCCAAAGAAAGGAGAAAAAGCTAAAGAAAGCAGGATGATGAAGTGGATAAAGCATTGACCT

FIG. 6. Alignment of the DNA sequences of the PCR product derived from the hybrid RNA detected in Vero cells following selective passage. The sequences consist of (A) the 5' and (B) the 3' ends of four independent clones (VPCR 1 to 4). The published sequences for the corresponding regions of Beaudette (top) and M41 (bottom) are included. Asterisks above the sequence show the positions where the two sequences differ. Nucleotide differences observed between the published M41 sequence and those observed from the M41-CK virus used in this work, as determined for the sequences in Fig. 3, are indicated with arrows. The potential crossover site, defined as the region in which the sequence derived from the PCR products changes from one parental strain to the other, is shown as a boxed region. Regions in which the sequence was not determined are indicated as dots; positions where the nucleotides were not certain are indicated by an N. The numbers above the sequences refer to the nucleotide positions for the complete sequence of the Beaudette genome. The lines at the 5' end of (A) and 3' end of (B) indicate the sequences of oligonucleotides B5 and M8, respectively, used to produce the PCR products.

also has a G at this position our previous sequence data showed that the CK adapted M41 (M41-CK) virus, used in this work, had an A residue at this position (Fig. 6A). The C residue at position 26,062 on the PCR product could have been derived either from the Beaudette sequence or the M41-CK virus. However, the C at position 26,067 on the PCR product corresponded to the Beaudette sequence because although the published M41 sequence also contained a C residue, we found that the M41-CK sequence consisted of a T residue at this position. The remainder of the residues at the 5' end of the PCR product that differed between Beaudette and M41 or M41-CK corresponded to the M41-CK sequence as did the 3' end of the PCR product (Fig. 6B). Interestingly, all four clones contained the same crossover site, indicating that there was either a bias in the PCR population or that there was only a small population of replicating recombinant viruses detectable within the region of the IBV genome under investigation. Analysis of the sequences showed that the crossover site between

the two IBV sequences occurred over a five-nucleotide region corresponding to nucleotides 26,068–26,072 on the Beaudette genome.

DISCUSSION

Our experiments have shown that recombination between two strains of IBV, leading to the production of infectious recombinant virions, occurred during the mixed infection of embryonated eggs. This is the first experimental demonstration of recombination in IBV. Previous sequencing studies had provided only circumstantial evidence that recombination occurs in field isolates of IBV (Cavanagh and Davis, 1988; Kusters *et al.*, 1989, 1990; Cavanagh *et al.*, 1992; Wang *et al.*, 1993, 1994; Jia *et al.*, 1995). For example, the S gene sequences of the Dutch isolate D207/78 and the UK isolate UK/6/82 are almost identical, >98%, until nucleotide 3315 after which the identity falls to only 57%. However, after nucleotide 3315 the nucleotide identity between the UK/6/82 isolate and another Dutch isolate, D1466/78, is 97%, whereas the pre-

ceding nucleotides in the S sequence show an identity of 73%. These observations suggested that the evolution of the UK/6/82 isolate involved a recombination event, near the end of the S gene, between strains resembling the Dutch isolates D207 and D1466 (Kusters *et al.*, 1990). However, there has previously been no direct experimental evidence that recombination can occur in IBV.

The technique of strain-specific PCR has been shown to be an extremely powerful method for distinguishing between closely related virus strains (Banner and Lai, 1991; Jarvis and Kirkegaard, 1992) and opens the possibility of detecting potential recombinants in the absence of selection. Our oligonucleotides were designed for their ability to distinguish between two IBV strains and could therefore be used for the detection of potential recombinants using strain-specific PCR. Although oligonucleotide M8 had only two nucleotide mismatches in relation to the corresponding Beaudette sequence, the oligonucleotide was shown to be specific for M41. This is in agreement with the observation that an oligonucleotide with three nucleotide mismatches at the 3' end was shown to be strain-specific for MHV using PCR (Baker and Lai, 1990). Previous studies on the effects of primer-template mismatches in PCR revealed that double nucleotide mismatches at the 3' end of an oligonucleotide drastically reduced the PCR product yield (Kwok *et al.*, 1990; Jarvis and Kirkegaard, 1992), indicating that PCR can differentiate between sequences with no more than two consecutive base mismatches.

Our oligonucleotides for distinguishing between two IBV strains gave rise to products of 970 and 1210 bp. Liao and Lai (1992) had previously shown that for the coronavirus MHV a region of 300 nucleotides was sufficient for recombination events, indicating that a distance of 970 or 1210 nucleotides in IBV should be sufficient for the detection of recombination events.

One of the disadvantages of using PCR for detection of potential recombinants is the fact that both reverse transcriptase and *Taq* polymerase are capable of producing recombinant RT-PCR products by switching between templates. One of our control RT-PCRs, using a simple mixture of M41 and Beaudette RNA, indicated that the hybrid RNAs produced following mixed infection had not arisen from template switching by either of these two enzymes. The result supported the proposal that the RT-PCR products derived from virion RNA isolated from viruses in the allantoic fluid of coinfecting eggs resulted from hybrid RNAs produced by the coronavirus polymerase.

Sequence analysis of cloned RT-PCR products, synthesized from virion RNA isolated from viruses in the allantoic fluid of coinfecting eggs, confirmed that they were derived from hybrid IBV RNAs. All the cloned PCR products sequenced consisted of regions derived from Beaudette- and M41-specific sequences and showed different crossover sites, demonstrating that a number of

recombination events had occurred. These recombination events seemed to have occurred randomly between the selected primers. Previous work, on MHV, had suggested that there might be favored recombination sites in coronavirus genomes (Makino *et al.*, 1986), although it is now believed that the apparent clustering of recombination sites resulted from the use of selection pressure in the experiments (Banner and Lai, 1991). To detect hybrid IBV RNAs, following as little selection pressure as possible, we used RT-PCR on virion RNAs produced from the mixed infections without passage. This method also allowed the detection of potential hybrid RNAs derived from recombinants with selective disadvantages that otherwise would be lost following any selection procedure(s).

An alternative explanation for the detection of hybrid RNA molecules in this study is that they were not derived from genomic RNAs but from subgenomic RNAs, as small amounts of subgenomic mRNAs were detected in IBV preparations (Zhao *et al.*, 1993). However, such molecules would still have been generated by recombination. Definitive proof that IBV can undergo recombination, resulting in the production of hybrid genomes, would require the demonstration that hybrid RNAs can be passaged. It was very unlikely that we would have been able to isolate a recombinant virus because the recombination event studied is unlikely to have a selective advantage over the parental strains and potential recombinant viruses could be out competed. We currently have no tools, e.g., temperature-sensitive (*ts*) mutants and/or appropriate monoclonal antibodies with which to select our recombinant viruses.

In order to demonstrate that the hybrid RNAs were able to replicate, we used a selective passage method (neutralization with a MAb, Vero cells) followed by strain-specific PCR. The procedure resulted in no IBV-M41 genomes being present in any of the cell extracts following passage in Vero cells of the progeny virus following mixed infections of embryonated eggs. This had two advantages. Firstly, removal of one parental strain would have reduced the chance of the parental viruses outgrowing recombinant viruses. Second, this removed the possibility that the hybrid RNA could have been produced as a result of some RT-PCR artefact. We demonstrated that a hybrid RNA, consisting of the Beaudette genome with M41 sequence at the 3' end, downstream of the Beaudette-specific oligonucleotide B5 site, was passaged from viruses present in the allantoic fluid of coinfecting embryonated eggs. This result demonstrated that a replicating hybrid RNA was produced as a result of the coinfection of embryonated eggs and confirmed, experimentally, that recombination can occur in IBV.

This demonstration shows that RNA recombination can occur in coronaviruses in addition to that previously published for MHV. The observation of recombination with IBV and MHV supports the notion that RNA recombi-

nation may be a common phenomenon within the *Coronaviridae* and may play an important role in the evolution and divergence of this group of viruses.

Unlike poliovirus, for which Kirkegaard and Baltimore (1986) showed that the most likely mechanism for recombination was template switching, the mechanism for recombination in coronaviruses has not been studied in such great detail. Based on the observation of incomplete transcripts (Baric *et al.*, 1987), it has been suggested that coronavirus recombination is also caused by template switching (Makino *et al.*, 1986; Lai, 1992). In this model the polymerase falls off the initial template, possibly caused by a region of high secondary structure in the RNA, anneals to another template and continues replication. We could not find a link between any potential strong secondary structures and the recombination sites identified in our recombinants. Nevertheless, recombination resulting from the nonprocessive nature of the coronavirus polymerase is the likely reason for the high frequency in coronaviruses.

Our demonstration of recombination in IBV opens new possibilities for the study of IBV. Recombinants could be used to study the virus on a molecular level as previously demonstrated for MHV (as reviewed by Lai, 1992) and also opens the possibility of making site-specific recombinants for studying the function of the virus gene products. Site-specific recombinants were isolated for MHV, without selection, using defective interfering RNA (van der Most *et al.*, 1992). Recently, we described a replicating defective RNA of IBV-Beaudette (Penzes *et al.*, 1994) and are currently investigating the possibility of using this and targeted RNA recombination for the production of recombinants to study the pathogenicity of IBV.

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