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Characterization of a Replicating and Packaged Defective RNA of Avian Coronavirus Infectious Bronchitis Virus

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The Beaudette strain of IBV was passaged 16 times in chick kidney cells. Total cellular RNA was analyzed by Northern hybridization and was probed with ^{32}P-labeled cDNA probes corresponding to the first 2 kb of the 5′ end of the genome, but excluding the leader, and to the last 1.8 kb of the 3′ end of the genome. A new, defective IBV RNA species (CD-91) was detected at passage 6. The defective RNA, present in total cell extract RNA and in oligo-$(dT)_{30}$-selected RNA from passage 15, was amplified by the reverse transcription-polymerase chain reaction (RT-PCR) to give four fragments. The oligonucleotides used were selected such that CD-91 RNA, but not the genomic RNA, would be amplified. Cloning and sequencing of the PCR products showed that CD-91 comprises 9.1 kb and has three regions of the genome. It contains 1133 nucleotides from the 5′ end of the genome, 6322 from gene 1b corresponding to position 12,423 to 18,744 in the IBV genome, and 1626 from the 3′ end of the genome. At position 749 one nucleotide, an adenine residue, was absent from CD-91 RNA. By Northern hybridization CD-91 RNA was detected in virions in higher amounts than the subgenomic mRNAs.

Infectious bronchitis virus (IBV) is an enveloped RNA virus of the genus *Coronavirus* in the family *Coronaviridae*. It has a nonsegmented, single-stranded, positive-sense RNA genome of 27.6 kb (Boursnell *et al.*, 1987). In infected cells the virus-encoded RNA-dependent RNA polymerase replicates the genome into a minus-sense RNA, which then serves as template for both synthesis of the genome RNA (gRNA) and transcription of the five subgenomic mRNAs (reviewed by Spaan *et al.*, 1988; Lai, 1990). The mRNAs form a 3′ coterminal nested set; that is, the sequence of each mRNA is contained entirely within the next larger mRNA. The 63 nucleotides at the 5′ terminus of each mRNA are derived from the 5′ end of the genome and form a leader sequence (Brown *et al.*, 1984). It has been proposed that coronavirus murine hepatitis virus (MHV) utilizes a free leader RNA species which serves as a primer for the transcription of subgenomic mRNAs (leader-primed transcription) (Baric *et al.*, 1985; Makino *et al.*, 1986). During transcription and replication the RNA polymerase may pause, fall off, and then rejoin the original or another RNA template. The discontinuous and nonprocessive nature of transcription may give rise to incomplete RNA intermediates (Baric *et al.*, 1985, 1987), defective RNAs (D-RNAs) (Makino *et al.*, 1984; van der Most *et al.*, 1991; Furuya *et al.*, 1993), and recombinants (Lai *et al.*, 1985; Keck *et al.*, 1988; Kusters *et al.*, 1989, 1990; Cavanagh *et al.*, 1992; Wang *et al.*, 1993).

D-RNAs are naturally occurring deletion mutants and have been described for many viruses (Barrett and Dimmock, 1986). Characteristically, D-RNAs lack part of the viral genome and can replicate only with the help of standard virus. To date, coronavirus D-RNAs have been reported only for MHV (Makino *et al.*, 1984; van der Most *et al.*, 1991).

In this article we report the cloning, sequencing, and characterization of a naturally occurring D-RNA, CD-91, of coronavirus IBV. This is the first description of a D-RNA of IBV which is replicated and packaged into virus particles.

MATERIALS AND METHODS

Virus and cells

Beaudette-US is an egg-adapted strain of IBV which, after passage in chick kidney (CK) cells, had been passaged 10 times in Vero cells without plaque purification (Alonso-Caplen *et al.*, 1984; Cavanagh *et al.*, 1986). The virus in this study had been passaged a further four times in Vero cells, twice in chick embryos, and once in CK cells. CK cells were prepared from 1-week-old Rhode Island Red chicks. Vero cells were obtained from Flow Laboratories (ATCC No. CCL 81). Both types of cells were

Sequence data from this article have been deposited with the EMBL, GenBank, and DDJB Nucleotide Sequence Databases under Accession No. Z30541.

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maintained at 37° in MEM–Eagle's medium (Flow Laboratories) supplemented with 0.29% tryptose phosphate broth (TPB), 0.2% bovine serum albumin (BSA), 0.02 *M* *N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES), 0.2% $NaHCO_3$, 0.01 *M* L-glutamine, and antibiotics, pH 6.8 (maintenance medium, MM).

Virus titration

Confluent CK cells in 96-well plates were washed with medium. The virus was diluted in 10-fold steps and 8 wells per dilution were infected with 100 μl. After 4 days of incubation at 37° in 5% CO_2 the cell sheet had been destroyed in virus-containing wells. The 50% tissue culture infective dose ($TCID_{50}$) was calculated according to the method of Reed and Muench (1938).

Undiluted passage of IBV-Beaudette

Confluent, 3-day-old CK cells in 25-cm^2 flasks were used for the undiluted passages. The growth medium was removed and the cell sheet was washed once with 3 ml of warm MM. Then 0.5 ml of undiluted virus was placed on the cell sheet and the flasks were gently rocked to distribute the inoculum. The cells were then incubated at 37° for 1 hr, with occasional rocking, after which the inoculum was removed and the cells were washed with 6 ml of MM. This was replaced with 8 ml MM and the cells were incubated at 37° for 24 hr. The supernatant was then harvested and stored at −70°. Some of the supernatant was further passaged in fresh cells.

Preparation of purified virions, cell extract, and viral RNAs

The medium from infected 25-cm^2 flasks was centrifuged at 1000 rpm (300 *g*) for 15 min in an H2000 rotor (Sorvall, RC3B) and the virus precipitated from the supernatant with an equal volume of saturated $(NH_4)_2SO_4$ at 4° overnight. The virus was then pelleted at 6000 rpm (5860 *g*) for 30 min in an HB4 rotor (Sorvall, RC5B). The virus pellet was resuspended in 1 ml of TNE solution (100 m*M* NaCl, 1 m*M* EDTA, 10 m*M* Tris–HCl, pH 7.4) and centrifuged through 25% w/v sucrose at 28,000 rpm (140,000 *g*) for 2 hr in a SW-40 Ti rotor (Beckman; Sorvall, OTD55B). Cell-associated RNA (from CK cells) and RNA from the pelleted virions were extracted using the guanidinium isothiocyanate method (Chomczynski and Sacchi, 1987). Some of the total cellular RNA was selected with paramagnetic oligo$(dT)_{30}$ particles according to the manufacturer's instructions (Scigen Ltd., Sittingbourne, UK).

Northern blot analysis

RNAs were separated in 1.2% agarose gels containing formaldehyde (Sambrook *et al.*, 1989) and the gel was exposed to uv light (302 nm) (Ultra Violet Products) for 2 min to nick the RNA sufficiently to facilitate the transfer of the large gRNA. The RNAs were transferred to Hybond C-Extra (Amersham International) by capillary blotting overnight. The filters were probed with various radiolabeled ([α-^{32}P]dCTP) cDNA probes covering different parts of the IBV-Beaudette genome (Fig. 1A). Probes 204, 210, 205, 220, 249, and 182 were prepared by restriction digests of IBV-Beaudette cDNA clones (Boursnell *et al.*, 1987) followed by gel purification of the Beaudette inserts. Probes "S1," "S2," 611, and "N," and the 3′ end probes were prepared by RT-PCR of the IBV-Beaudette genome. cDNA synthesis of the 5′ end probe was primed with oligonucleotide 42 (5′-CCTCTCCAAGCACTGCTG) complementary to positions 2014–1997 on the IBV-Beaudette genome. The cDNA was then amplified by PCR using oligonucleotides 42 and 93/102 (5′-GCTGGTCCTCATAGGTGTTC) which corresponds to positions 85–104 in the genome. The radiolabeled probes were made by the random hexanucleotide primer method (Sambrook *et al.*, 1989).

RT-PCR amplification of CD-91 RNA

CD-91 RNA present in total cellular RNA and in oligo$(dT)_{30}$-selected RNA from passage 15 was amplified by RT-PCR in four separate fragments. The cDNA synthesis of the four fragments was primed with oligonucleotides 21 (5′-GAGTCTTTGCCCGGGTCGAAATGTCTCAAAGC, 12,733–12,714), 93/118 (5′-CCTGGTTTTTCCTGGTTCCC, 15,650–15631), 93/104 (5′-GGCGTCTCCAGTATCCATG, 26092–26074) and oligo$(dT)_{18}$-*Not*I (5′-AACTGGAAGAATTCGCGGCCGCAGGAAT$_{18}$) (Fig. 1). The underlined letters in the oligonucleotides indicate restriction sites and non-IBV sequences. The reaction conditions were 2 μl 20 m*M* $MgCl_2$, 2 μl 0.1 *M* DTT, 4 μl 5× RT buffer (250 m*M* Tris–HCl, pH 8.3, 375 m*M* KCl, 15 m*M* $MgCl_2$), 2 μl 10 m*M* dNTP, 10 pmol oligonucleotide, 200 U Superscript RT (BRL) incubated at 42° for 90 min. Four microliters of the resulting cDNA was then amplified by the PCR with oligonucleotides 43 (5′-GGGCCCACTTAAGATAGATATTAATATA, 1–22) and 21, 93/116 (5′-AAGGCACTGGCTATTTTTGAG, 1111–1131) and 93/118, 93/117 (5′-TGCTAGTGCAGAAGTGCGAG, 15,578–15,597) and 93/104, and 35 (5′-GAGTCTTGCCCGGGCAGATTCGCAGCATGCAC, 16,785–16,803) and oligo$(dT)_{18}$-*Not*I to obtain the four fragments of CD-91 RNA. The amplification reaction conditions were 5 μl 10× *Taq* buffer (500 m*M* KCl, 100 m*M* Tris–HCl, pH 8.0, 100 m*M* NaCl, 0.1 m*M* EDTA, 5 m*M* DTT, 1% Triton X-100), 2.5 μl 25 m*M* $MgCl_2$, 10 pmol of each oligonucleotide, 1 μl 10 m*M* dNTP, 4 μl cDNA, 2.5 U *Taq* polymerase (Promega) at 94° for 45 sec, 52° for 30 sec, and 72° for 3 to 6 min 30–35 times in an Omnigene thermal cycler (Hybaid Ltd., Middlesex, UK).

Cloning and sequencing

The amplified CD-91 fragments were blunt-ended and cloned into the *Sma*I site of pBluescript II SK(+) (Stra-

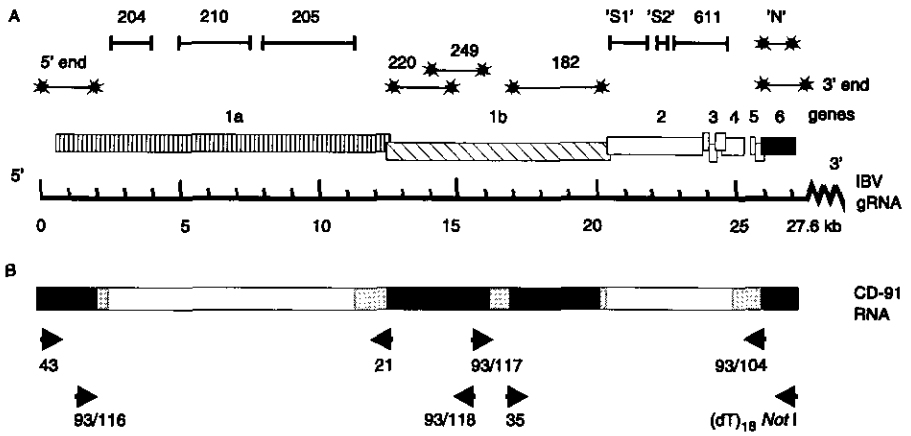

FIG. 1. (A) Tentative mapping of CD-91 by Northern hybridization. CD-91 RNA was electrophoresed in agarose gel and probed with various ^{32}P-labeled DNA fragments (0.4–3.2 kb) covering most of the IBV-Beaudette genome. The genomic 5′ end probe, probes 220, 249, 182, and "N," and the genomic 3′ end probe hybridized to the CD-91 RNA and gave strong signals on the autoradiograph (marked * — *). Probes 204, 210, 205, "S1," "S2," and 611 did not hybridize to CD-91 RNA. (B) Diagram showing the putative sequences of CD-91 RNA as deduced from the hybridization results. The parts of the genome that hybridized with the probes and were potentially present in CD-91 RNA are shown in black. The gray regions are areas that could have been present in CD-91, the white regions corresponding to sequences of the probes that were unable to detect CD-91. The positions of the oligonucleotides that were subsequently used to amplify four fragments of CD-91 RNA are also shown.

tagene) using standard cloning procedures (Sambrook *et al.*, 1989) or cloned into pCR vector (Invitrogen) according to the manufacturer's instructions. The clones were sequenced on a 373A DNA sequencer (Applied Biosystems Inc.) using the PRISM Ready Reaction Dye-Deoxy Terminator cycle sequencing kit according to the manufacturer's instructions (Applied Biosystems Inc.). Sequence data were compiled using the Staden package (Staden, 1986) and analyzed by the Gene Runner package (Hastings Software Inc., U.S.A.).

RESULTS

Detection of a defective IBV RNA in CK cells

IBV Beaudette-US was passaged undiluted in CK cells 16 times. At 24 hr postinfection (p.i.) total cellular RNA was extracted and analyzed by Northern hybridization using ^{32}P-labeled cDNA probes corresponding to the 3′ 1.8 kb of the Beaudette-US genome (3′ probe) (Fig. 2A). Due to the nested set structure of the IBV mRNAs, the 3′ probe hybridized to all IBV RNA species. A new RNA species, CD-91, appeared at passage 6 which was larger than mRNA 2 and persisted in high amounts during subsequent passages. To determine whether CD-91 RNA had 5′ genomic sequence, the blot was reprobed with a ^{32}P-labeled cDNA probe corresponding to the first 2 kb of the genome (5′ probe) (Fig. 2B). To avoid detection of the subgenomic mRNAs, the 5′ probe lacked the leader sequence which is present at the 5′ end of all coronavirus mRNAs. The 5′ probe hybridized to the genomic RNA and also detected CD-91 RNA. $TCID_{50}$ analysis showed no fluctuation of infectious virus titer, all passages having a $TCID_{50}$ between $10^{7.5}$ and 10^{8} (data not shown).

IBV Beaudette-US was also passaged in Vero cells 17 times, as described for CK cells. However, no putative defective RNAs with genomic 5′ and 3′ sequences were detected by Northern hybridization (results not shown).

Mapping and sequencing of defective RNA CD-91

In order to determine which genomic sequences were present in the CD-91 RNA, total RNA from IBV-infected cells was probed with various ^{32}P-labeled cDNA probes which covered most of the Beaudette genome (Fig. 1A). The cDNA probes used varied in size between 0.4 and 3 kb. The 5′ probe, and probes 220, 249, 182, the N gene probe, and the 3′ end cDNA probe hybridized to CD-91 RNA, whereas probes corresponding to gene 1a (204, 210, and 205) in the IBV genome and the S, M, and 3a, 3b, and 3c genes ("S1," "S2," and 611) did not (Fig. 1A). The results obtained indicated that in addition to the genomic 5′ and 3′ sequences, CD-91 RNA contained an internal region, corresponding to approximately the first 6 kb of gene 1b. Gene 1a was found to be almost absent and none of the structural genes, except some of N gene, were present (Fig. 1B).

Based on gel electrophoretic mobility, the size of CD-91 RNA was estimated to be about 9 kb. Due to this large size, CD-91 RNA could not be amplified by PCR using primers specific for the ends of the genome. An alternative strategy was chosen by which 5′ and 3′ ends of CD-91 RNA and the middle region, corresponding to gene 1b, were amplified separately with four different oligonucleotide pairs, as described under Materials and Methods (Fig. 1B). Each of the four overlapping PCR fragments (5′ to 3′: 1.5, 3.2, 3.2, and 3.6 kb) contained one of the two putative rearrangement sites of CD-91 RNA. To identify possible mutations caused by reverse tran-

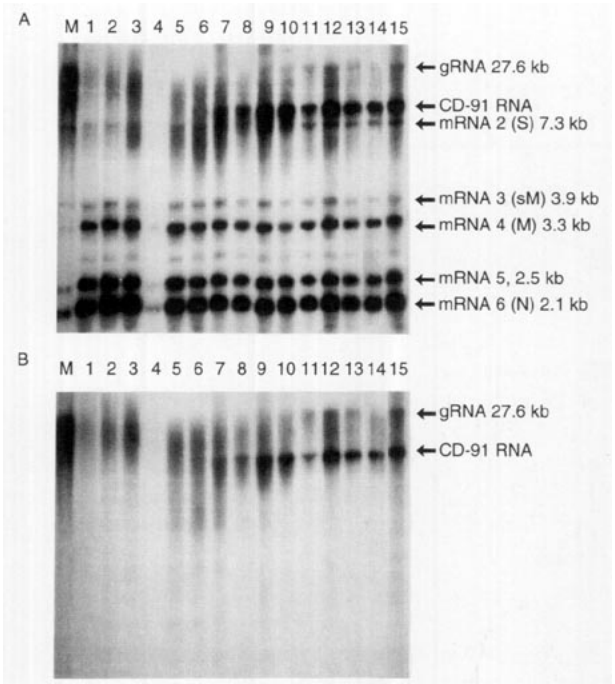

FIG. 2. Northern blot of IBV Beaudette-US RNAs from CK cells. Beaudette-US was passaged undiluted in CK cells (passage 1 to 15) and the total cell extract RNA was separated in denaturing agarose gels and probed with a ^{32}P-labeled genomic 3′ end 1.8-kb probe (A) or with a genomic 5′ end 2-kb probe (B). Lane M is IBV-Beaudette RNA extracted from purified virions which also contain subgenomic mRNAs (Zhao *et al.*, 1993), used as a marker. Lanes 1–15 show the undiluted passages of Beaudette-US, where a new RNA species, CD-91, appeared at passage 6 and persisted in high amounts during subsequent passages. The mRNAs are labeled according to the recommendations of the Coronavirus Study Group (Cavanagh *et al.*, 1990). The amount of RNA in lane 4 was low due to a technical error.

scriptase or *Taq* polymerase, each RT-PCR reaction was repeated at least twice. The PCR products, representing the four overlapping fragments of CD-91 RNA, were cloned and at least two clones from independent RT-PCR reactions sequenced on an ABI 373A DNA sequencer. The sequencing data confirmed that CD-91 RNA was composed of three regions from the IBV genome (Fig. 3A), 1.1 kb from the 5′ end of the genome, 6.3 kb of gene 1b, and 1.6 kb from the 3′ end of the IBV genome. With one exception, the sequence of CD-91 RNA corresponded to the equivalent region of the published IBV-Beaudette sequence (Boursnell *et al.*, 1987): at position 749, near the 5′ end, an adenine residue was absent from CD-91 RNA (Fig. 4). To confirm that the adenine residue deletion in CD-91 RNA was not an artifact, several RT-PCR reactions were performed using RNA from passages 1 (see below), 2, 7, and 15. Cloning and sequencing of the PCR products confirmed that the adenine residue deletion was not an artifact. Using total RNA from infected cells (passage 7) the corresponding region of the gRNA was amplified, cloned, and sequenced. The results confirmed the presence of the adenine residue in the gRNA (Fig. 4).

CD-91 RNA has one long open reading frame (ORF), nucleotides 996 to 7463, corresponding to nucleotides 997–1133, 12,423–18,744, and 25,983–25,990 in the IBV genome. Due to the adenine residue deletion, this frame is 467 nucleotides shorter at its 5′ end compared to IBV gene 1a. This CD-91 RNA ORF stops after the 3′ rearrangement site at nucleotide 7463 on CD-91 RNA. A second ORF corresponding to the 3′ half of the N gene is also present in CD-91 RNA. It comprises nucleotides 8009 to 8575, corresponding to nucleotides 26,536–27,102 in the genome.

Detection of CD-91 RNA in early passages in CK cells

To investigate whether CD-91 RNA was present earlier than passage 6, in which it was first detected by Northern hybridization, the 5′ 1a–1b rearrangement site of CD-91 was selectively amplified by RT-PCR from total RNA from infected CK cells from passage 0 (starting inoculum, working stock virus), 1–7, 15, and 16 (undiluted virus passages) and from Beaudette-US Vero passage 2. The 5′ 1a–1b join region was amplified with oligonucleotides 93/102 and 21 and yielded the expected 1.3-kb product for CD-91 RNA from passage 4 (Fig. 5A). However, from passages 1 to 6 a larger PCR fragment, 1.5 kb, was detected (Fig. 5A). The DNA from passages 1 and 2 was sequenced and found to originate from the mispriming of oligonucleotide 21 around position 1602 in the genome. As the number of CD-91 RNA molecules increased, around passage 4, oligonucleotide 21 started to preferentially anneal to CD-91 RNA, rather than to the genome RNA, that was present in a higher proportion in passages 1 to 3. To further increase the sensitivity of the PCR assay, the PCR products corresponding to the 1a–1b rearrangement site from passages 1 to 6 were reamplified with nested oligonucleotides 93/106 (5′-GGCAGAAGTTTGACCGTAG, 674–692) and ST4 (5′-CTAGCGCAGTTACGCTTCAA, 12,490–12,471). The expected 0.5-kb DNA, corresponding to the 1a–1b rearrangement site on the CD-91 RNA, was detected in all passages starting from passage 1, but not in either CK passage 0 or in Beaudette-US Vero passage 2 (Fig. 5B). The specificity of the 0.5-kb DNA has been confirmed by cloning and sequencing of the PCR products from passages 1, 2, 7, and 15. All the clones from passages 2, 7, and 15 contained the 5′ 1a–1b rearrangement site of CD-91 RNA and lacked the adenine residue at position 749 (CD-91-like clones). However, out of 10 clones sequenced from passage 1, only a minority of them were CD-91-like. Four different types of clones were identified with a 1a–1b rearrangement site which was shifted about 100 to 400 nucleotides toward the 5′ end of the genome, when compared to the CD-91 RNA 5′ rearrangement site. None of these different types of clones lacked the adenine residue at position 749.

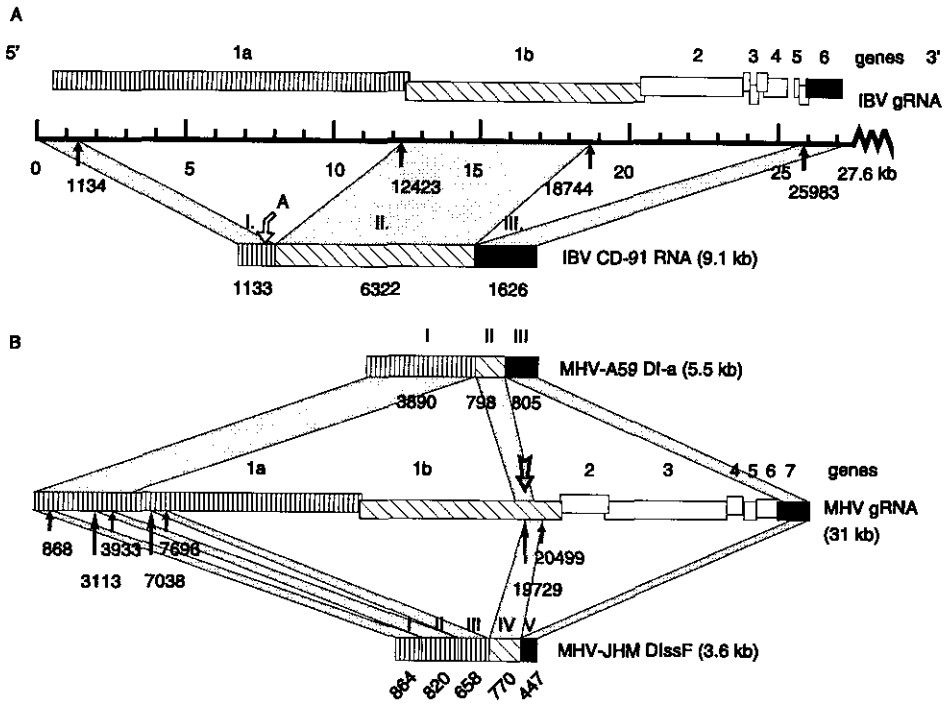

FIG. 3. (A) Diagram showing the structure of IBV CD-91 RNA. CD-91 RNA contains three regions from the IBV genome: 1133 nucleotides from the 5' end of the IBV-Beaudette genome (region I), 6322 nucleotides from gene 1b (region II), and 1626 nucleotides from the 3' end of the genome (region III). At position 749 an adenine residue deletion was found in CD-91 RNA (marked with open arrow). (B) Diagram showing the regions of MHV-A59 DI-a (van der Most *et al.*, 1991) and MHV-JHM DIssF (Makino *et al.*, 1990). The MHV packaging signal is marked with an open arrow (Fosmire *et al.*, 1992). The solid arrows indicate the positions of the rearrangement sites of the different D-RNAs on the IBV and MHV genomes.

Packaging of CD-91 RNA

To study whether CD-91 RNA was packaged into virions, RNAs extracted from purified virions at passage 0 and from CD-91-containing virions from passage 15 were analyzed by Northern hybridization using a ^{32}P-labeled N gene cDNA probe (Fig. 6). This showed that virus preparations contained more CD-91 RNA than subgenomic mRNAs, which have previously been shown to be packaged (Zhao *et al.*, 1993; see also Figs. 2 and 4, lane M). CD-91 RNA was not detected in passage 0 by Northern hybridization (Fig. 6). The molar ratio of CD-91 RNA compared to the viral mRNAs in IBV-Beaudette virus preparations was estimated from the amount of N gene probe bound to the Northern blots as described by Zhao *et al.* (1993). The amount of CD-91 RNA was found to be about 2.3-fold greater than mRNA 6, the most abundant subgenomic mRNA in virions (Zhao *et al.*, 1993). Due to transfer difficulties and degradation, the gRNA was only detected in low amounts in both passages 0 and 15.

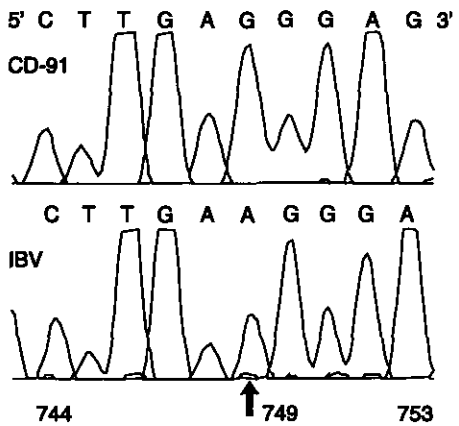

FIG. 4. Chromatogram of the nucleotide sequence of CD-91 and IBV-Beaudette cDNA, nucleotides 744 to 753, obtained from an ABI 373A DNA sequencer. CD-91 RNA and IBV gRNA were amplified by RT-PCR with different oligonucleotides using total cellular RNA from IBV-Beaudette passage 7. At position 749 the gRNA has an adenine residue which was absent from CD-91 RNA.

DISCUSSION

This study reports the detection, cloning, sequencing, and characterization of CD-91 RNA, a 9.1-kb defective RNA of coronavirus IBV. CD-91 RNA of IBV Beaudette-US was detected after undiluted passage in CK but not Vero cells. The defective RNA was not detected in CK cell passage 0 or in Vero cell passage 2, even by nested-set PCR, whereas CD-91 was detected by this method starting at CK cell passage 1. The finding that CD-91 RNA was not detected in the Vero cell passages and that heterogeneous defective RNAs were detected in CK cell passage 1 suggests that CD-91 RNA may have been generated in CK passage 1 and subsequently became the predominant D-RNA species. However, CD-91 RNA

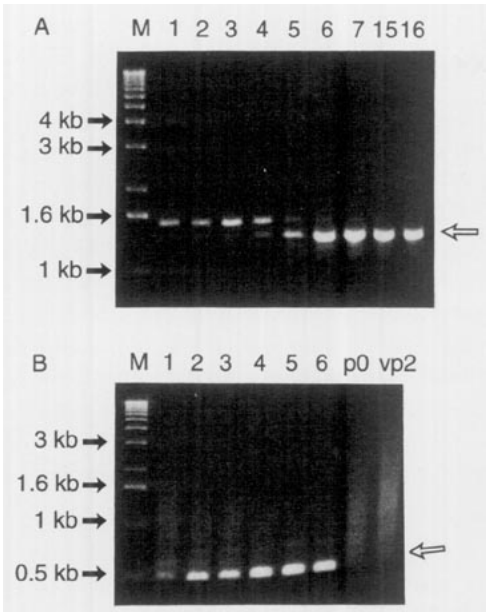

FIG. 5. Detection of CD-91 RNA in early passages by nested-set PCR. (A) The 5′ end of CD-91 RNA, containing the 1a–1b rearrangement region, from passages 1 to 7, 15 and 16 was amplified by RT-PCR with oligonucleotides 93/102 and 21. The expected 1.3-kb fragment, which included the region where the adenine residue was deleted (see Fig. 3A), was detected from at least passage 4 in increasing amounts (open arrow). The origin of the 1.5-kb fragment detected in decreasing amounts up to passage 6 was investigated and was found to be irrelevant to CD-91 RNA (see Results). (B) To further investigate the origin of CD-91 RNA the PCR products (A) from passages 1 to 6, from passage 0, and from Beaudette-US Vero passage 2 were reamplified by PCR using nested oligonucleotides 93/106 and ST4. A 0.5-kb fragment representing the 5′ 1a–1b rearrangement region of CD-91 RNA was detected in increasing amounts from passage 1. No fragments were detected by nested-set PCR in passage 0 (lane p0) or Beaudette-US Vero passage 2 (lane vp2).

replicated efficiently when Vero cells were infected with CD-91 RNA-containing CK cell passage 15 inoculum, supporting this hypothesis. However, the possibility that a pool of minute amounts of D-RNAs was already present in passage 0 cannot be excluded. It has been observed that the generation of DIs from several viruses was dependent on the cell type used (Kang and Allen, 1978; Kang *et al.*, 1981). Host cell factors may have played a role in the generation and accumulation of CD-91 RNA in CK cells but not in Vero cells. The detection of minute amounts of CD-91 or related RNA in higher Vero cell passages was not investigated. But, if it was present, CD-91 RNA was not detectable by Northern hybridization.

Cloning and sequencing of CD-91 RNA revealed that it comprised three discontinuous regions of the IBV genome (Fig. 3A); it is therefore similar in primary structure to MHV-defective interfering RNAs (DIs) (Makino *et al.*, 1985, 1988, 1990; van der Most *et al.*, 1991). However, CD-91 RNA is considerably larger (9.1 kb) than the MHV DIssF (3.6 kb, Makino *et al.*, 1990) or DI-a or DI-b (5.5 and 6.5 kb, respectively; van der Most *et al.*, 1991) (Fig. 3B). CD-91 RNA includes most of gene 1b (6.3 kb), but lacks the 3′ end of gene 1b that is present in the MHV DIs and where a 61-nt MHV packaging signal has been identified (Makino *et al.*, 1990; van der Most *et al.*, 1991; Fosmire *et al.*, 1992). If CD-91 RNA does contain a packaging signal then it has a different location than in MHV (Fig. 3B). This would agree with the observation of Fosmire *et al.* (1992) that the location of the packaging signal may differ among coronaviruses. The 5′ end gene 1a region of CD-91 RNA is smaller (1.1 kb) than the MHV gene 1a region present in the MHV DIs (1.6 to 3.9 kb) and lacks the 0.2-kb region of the MHV gene 1a that is located about 3.2 kb from the 5′ end of the MHV genome (Lee *et al.*, 1991) and which has been found to be necessary for MHV DI replication (Lin and Lai, 1993; Kim *et al.*, 1993b). Since CD-91 RNA is replicated efficiently in CK cells, we conclude that this region may not be required for the replication of CD-91 RNA, or that a homologous region may be located elsewhere.

CD-91 RNA has a single adenine residue deleted at position 749 (Fig. 4), confirmed in passages 1, 2, 7, and 15, which did not appear to adversely affect the stability of CD-91 RNA. Recently, Kim *et al.* (1993a) constructed a DIssE mutant (NE-1) that had an adenine residue deleted at position 376, resulting in the truncation of the long ORF. Mutant NE-1 was replicated less efficiently in the presence of the wild-type DIssE. The deletion was restored by RNA recombination with helper virus or by "RNA editing," i.e., an insertion of an adenine resi-

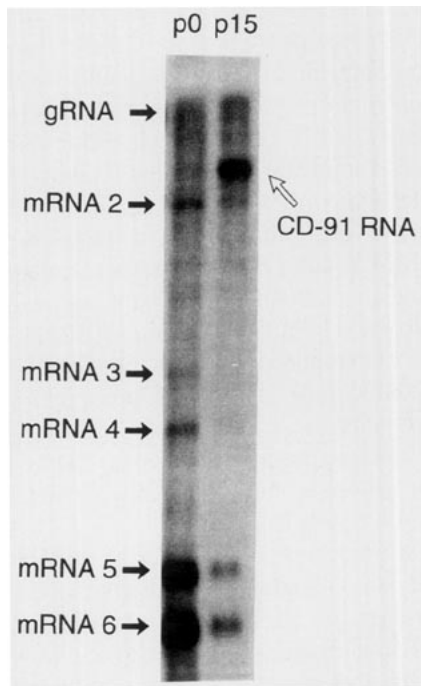

FIG. 6. Detection of CD-91 RNA in IBV virions by Northern hybridization. RNA from purified virions from passage 0 and 15 was extracted and electrophoresed on a formaldehyde agarose gel and probed with a ^{32}P-labeled cDNA probe corresponding the IBV N gene. No CD-91 RNA was detected in passage 0 (lane p0), whereas high amounts were present in the passage 15 virions (lane p15).

due nine nucleotides upstream of the deletion restored the ORF.

CD-91 RNA does not have one long ORF that spans the whole sequence. This is in contrast to DIssE (Makino *et al.*, 1988) and DI-a (van der Most *et al.*, 1991) in which the regions of the genome comprising the DIs are joined in one frame. The ORF of DI-a has been shown to be essential for replication (de Groot *et al.*, 1992). Due to the "A" deletion at 749, the IBV gene 1a ORF is truncated, spanning only nucleotides 529 to 765. However, due to the in-frame 1a–1b junction and the long (6.3 kb) gene 1b region (Fig. 3A), CD-91 RNA does have a long ORF, potentially encoding a protein of deduced M_r 244,000 that would include most of gene 1b. Since the CD-91 RNA 3′ 1b–N junction is out of frame, only the carboxy-terminal half of the N protein would possibly be translated from a separate ORF. At present it is not known which, if any, of these ORFs is essential for the replication of CD-91 RNA.

We did not observe a decrease in virus titer or in the amounts of subgenomic RNAs during undiluted virus passage. This was probably because we were unable to achieve high multiplicity of infection. Zhao *et al.* (1993) have calculated that approximately 1 in 24 virus particles of IBV-Beaudette contained mRNA 6. We have estimated that virus preparations contained approximately 2.3-fold more CD-91 than mRNA 6, i.e., about 1 in 10 virus particles would contain CD-91 RNA. Even if all the CD-91 molecules were present in virus particles that also contained gRNA, most cells would have been infected with virions containing gRNA but not CD-91. Hence the interfering property of CD-91 RNA would be masked by normal replication of gRNA in the majority of cells.

The amount of CD-91 in CK cell extracts (passage 9 onward) was somewhat greater than that of mRNA 4 and slightly less than mRNA 6 (Fig. 2). Zhao *et al.* (1993) calculated that the molar ratios of mRNA 4/gRNA and mRNA 6/gRNA in cell extracts are approximately 2.2 and 7.7, respectively, indicating that in the cell extracts as a whole there was about fivefold more CD-91 RNA than gRNA. This observation indicates that CD-91 was replicated more efficiently than gRNA, presumably because it is only one-third the size of gRNA. However, it was not possible to deduce the extent to which CD-91 replicated better than gRNA or how efficiently CD-91 RNA was packaged.

There is a great deal of evidence to support the view that discontinuous, leader-primed transcription is the primary and major mechanism whereby coronavirus subgenomic mRNAs are generated (Jeong and Makino, 1992, 1994; Yokomori *et al.*, 1992). However, there may also be a secondary mechanism for generating subgenomic mRNAs. Negative-sense RNAs containing anti-leader sequence have been demonstrated in cells infected with coronavirus transmissible gastroenteritis virus (Sethna *et al.*, 1989, 1991). This has led to the view that coronavirus subgenomic mRNAs can function as replicons. Supporting evidence has been obtained with MHV (Sawicki and Sawicki, 1990) and bovine coronavirus (Hofmann *et al.*, 1990). The potential of IBV subgenomic mRNAs to function as replicons has not been investigated. In our experiments cell extracts contained almost as much CD-91 RNA as mRNA 6, the most abundant mRNA, even though only a minority of infected cells would have contained CD-91 whereas the mRNAs would have been present in all the infected cells. If only a minority of the subgenomic mRNAs are generated by replication, our results suggest that CD-91 RNA replicates much more efficiently than the subgenomic mRNAs. This suggests that parts of the gene 1a and/or 1b sequences present in CD-91 RNA are required for efficient IBV RNA replication.

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