GENERATION OF A DEFECTIVE RNA OF AVIAN CORONAVIRUS INFECTIOUS BRONCHITIS VIRUS (IBV)*

Defective RNA of Coronavirus IBV

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ABSTRACT

The Beaudette strain of IBV was passaged 16 times in chick kidney (CK) 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 six 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 12423 to 18744 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

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INTRODUCTION

In infected cells the IBV-encoded RNA-dependent RNA polymerase replicates the genome into a minus-sense RNA, which then serves as template for synthesis of both the genome RNA (gRNA) and transcription of the five subgenomic mRNAs. During transcription and replication the RNA polymerase may pause, fall off and then rejoin the original or another RNA template. The discontinuous and non-processive nature of transcription may give rise to incomplete RNA intermediates (1, 2) defective RNAs (D-RNAs) (7, 12, 17) and recombinants (4, 10). To date, coronavirus D-RNAs have been reported only for MHV (12, 17). In this article we report the cloning, sequencing and characterization of a naturally occurring replicating and packaged D-RNA, CD-91, of coronavirus IBV.

MATERIALS AND METHODS

Virus and Cells, Undiluted Passage of IBV-Beaudette

Beaudette-US, an egg-adapted strain of IBV, chick kidney (CK) cells prepared from one-week-old Rhode Island Red chicks and Vero cells were used throughout the experiments. IBV-Beaudette was passaged undiluted in confluent CK and Vero cells at 37ºC for 24 h.

Preparation of Purified Virions, Cell Extract and Viral RNAs

Cell-associated RNA (CK-cells) and RNA in pelleted virions was extracted using the guanidinium isothiocyanate method. 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

Viral and cell-extract RNA was analyzed and radiolabeled probes ([α-^{32}P]dCTP) were prepared as described previously (18). The filters were probed with various radiolabeled cDNA probes covering different parts of the IBV-Beaudette genome.

RT-PCR Amplification, Cloning and Sequencing 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 (Superscript RT, BRL) of the four fragments was primed with oligonucleotides 21 (position in the IBV-Beaudette genome: 12733-12714), 93/118 (15650-15631), 93/104 (26092-26074) and oligo-$(dT)_{18}$-*Not*I. Four µl of the resulting cDNA was then amplified by the PCR (*Taq* polymerase, Promega) with oligonucleotides 43 (1-22) and 21; 93/116 (1111-1131) and 93/118; 93/117 (15578-15597) and 93/104 and with 35 (16785-16803) and oligo-$(dT)_{18}$-*Not*I to obtain the four fragments of CD-91 RNA.

The amplified CD-91 fragments were cloned into the *Sma*I site of pBluescript II SK(+) (Stratagene) using standard cloning procedures 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 DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems Inc.).

RESULTS

Detection of a Defective IBV RNA in CK Cells

IBV Beaudette-US was passaged undiluted in CK cells 16 times. At 24 h post infection (p.i.) total cellular RNA was extracted and analyzed by Northern hybridization using ^{32}P-labeled cDNA probes corresponding to the 5′ (excluding the leader) and the 3′ end of the genome. A new RNA species, CD-91, appeared at passage 6 which was larger than mRNA 2 (Fig. 1). $TCID_{50}$ analysis showed no fluctuation of infectious virus titer, all passages having a $TCID_{50}$ between $10^{7.5}$ - $10^{8.0}$.

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. The results 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. 2A).

CD-91 RNA was amplified by RT-PCR such that the 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 in Materials and Methods. Each of the four overlapping PCR fragments (5′ to 3′: 1.5 kb, 3.2 kb, 3.2 kb and 3.6 kb) contained one of the two putative rearrangement sites of CD-91 RNA. Cloning and sequencing of the PCR products confirmed that CD-91 RNA was composed of three regions from the IBV genome, as shown in Fig. 2A. With one exception, the sequence of CD-91 RNA corresponded to the equivalent region of the published IBV-Beaudette sequence (3): at position 749, near the 5′ end, an adenine residue was absent from CD-91 RNA. The adenine residue deletion in CD-91 RNA was confirmed on RNA from passages 1 (see below), 2, 7 and 15. Using total RNA from infected cells (passage 7) the corresponding region of the gRNA was also sequenced and the results confirmed the presence of the adenine residue in the gRNA.

CD-91 RNA has one long open reading frame (ORF) nucleotides 996 to 7463, corresponding to nucleotides 997-1133, 12423-18744 and 25983-25990 in the IBV genome. Due to the adenine residue deletion, this frame is 467 nucleotides shorter at its 5′ end when 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

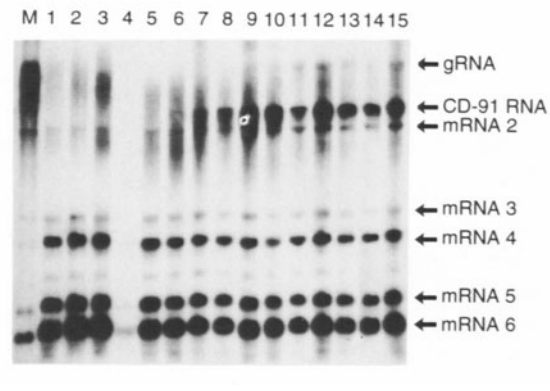

Figure 1. Northern blot of IBV Beaudette-US RNAs from CK cells. Beaudette-US was passaged undiluted in CK cells (passage 1 to 15) and the total cell extract RNA was separated in denaturing agarose gels and probed with a ^{32}P-labeled genomic 3′ end 1.8 kb probe. Lane M is IBV-Beaudette RNA extracted from purified virions which also contain subgenomic mRNAs, used as a marker. Lanes 1-15 show the undiluted passages of Beaudette-US, where a new RNA species, CD-91, appeared at passage six and persisted in high amounts during subsequent passages.

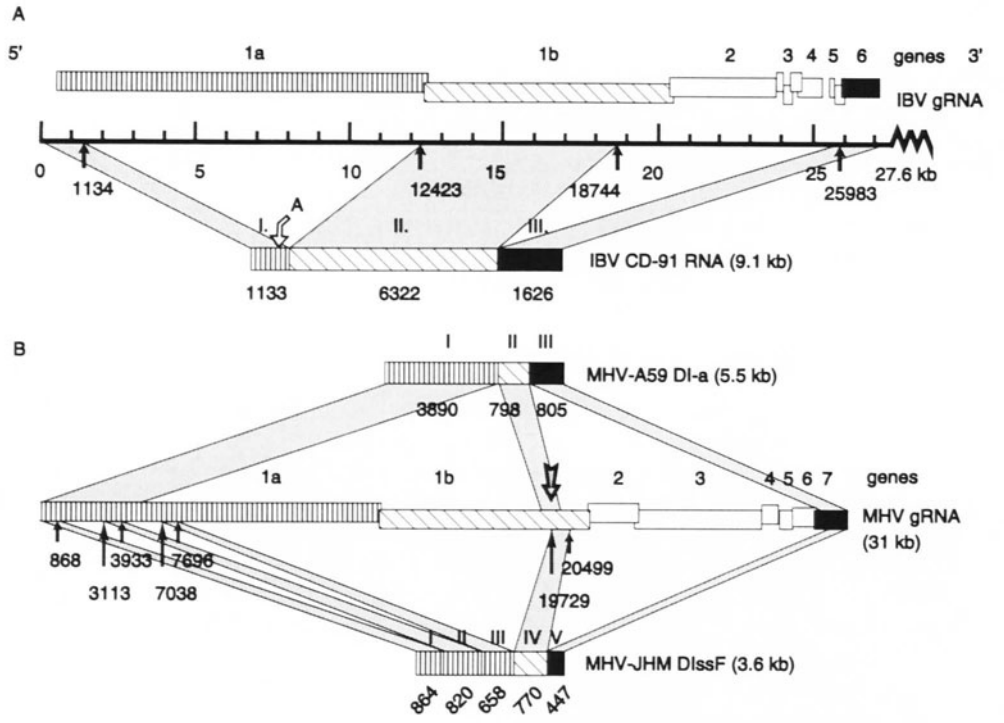

Figure 2. A. Diagram showing the structure of IBV CD-91 RNA. CD-91 RNA contains three regions from the IBV genome: 1133 nucleotides from the 5′ end of the IBV-Beaudette genome (region I), 6322 nucleotides from gene 1b (region II) and 1626 nucleotides from the 3′ end of the genome (region III). At position 749 an adenine residue deletion was found in CD-91 RNA (marked with unfilled arrow). **B.** Diagram showing the regions of MHV-A59 DI-a (17) and MHV-JHM DIssF (15). The MHV packaging signal is marked with an unfilled arrow (6). The solid arrows indicate the positions of the rearrangement sites of the different D-RNAs on the IBV and MHV genomes.

is also present in CD-91 RNA. It comprises nucleotides 8009 to 8575, corresponding to nucleotides 26536-27102 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), 1-7, 15, 16 and from Beaudette-US Vero passage 2. Use of oligonucleotides 93/102 and 21 yielded the expected 1.3 kb product for CD-91 RNA starting from passage 4. Then, each PCR product was re-amplified with nested oligonucleotides 93/106 (674-692) and ST4 (12490-12471) and yielded a 0.5 kb DNA fragment starting from passage 1. 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 ten clones sequenced from passage 1, only a minority of them was CD-91-like. Four different types of clones were identified with a 1a-1b rearrangement site which was shifted about 100 to 400 nucleotides towards 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.

Packaging of CD-91 RNA

To study whether CD-91 RNA was packaged into virions, RNAs extracted from purified virions at passage 0 and 15 were analyzed by Northern hybridization. The results showed that only virus preparations from passage 15 contained 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, the possibility that a pool of minute amounts of D-RNAs were already present in passage 0 cannot be excluded.

Cloning and sequencing of CD-91 RNA revealed that it comprised three discontinuous regions of the IBV genome (Fig. 2A); it is therefore generally similar to MHV defective interfering RNAs (DIs) (13, 14, 15, 17). However, CD-91 RNA is considerably larger (9.1 kb) than the MHV DIssF (3.6 kb) or DI-a or DI-b (5.5 kb and 6.5 kb (Fig. 2B). CD-91 lacks the 3′ end of gene 1b that is present in the MHV DIs and where a 61 nucleotide MHV packaging signal has been identified (6). If CD-91 RNA does contain a packaging signal then it has a different location than in MHV (Fig. 2B). 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 and which has been found to be necessary for MHV DI replication (9, 11). 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 does not have one long ORF that spans the whole sequence. This is in contrast to DIssE and DI-a 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 (5). Due to the 'A' deletion at 749, confirmed in passages 1, 2, 7 and 15, 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. 2A), 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.* (18) 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 onwards) was somewhat greater than that of mRNA 4 and slightly less than mRNA 6 (Fig. 1). Zhao *et al* (18) calculated that the molar ratios of mRNA 4/gRNA and mRNA 6/gRNA in cell extracts is approximately 2.2 and 7.7, respectively, indicating that in the cell extracts as a whole there was about 5-fold 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 of the size of gRNA.

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 (1, 2, 8). 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 (16). This has led to the view that coronavirus subgenomic mRNAs can be replicated. 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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