

TRANSCRIPTIONAL REGULATION OF RNA3 OF INFECTIOUS BRONCHITIS VIRUS

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1. INTRODUCTION

One of the unique properties of *Nidovirus* replication is production of a nested set of subgenomic RNAs by an unusual discontinuous transcription process.¹ These subgenomic RNAs are subsequently translated into viral proteins; regulation of subgenomic RNA transcription thus controls the timing and levels of viral protein expression during viral replication. Coronaviruses, which belong to the *Nidovirales* order, produce 6 to 9 subgenomic RNAs, depending on the virus. One of the established subgenomic RNA transcription regulation models for coronavirus involves sequences referred to as transcription regulation sequences (TRS). The TRS is well conserved among same strains of viruses, is located upstream of each open reading frame, and regulates subgenomic RNA production.² The TRS also has high sequence homology with the 3' end of the leader sequence at the 5' end of the genome, and the sequence homology between the body and leader TRS is an important factor regulating subgenomic RNA transcription.³ Studies with transmissible gastroenteritis virus showed that not only the homology between leader and body TRS is critical, but also sequences flanking the TRS contribute to the transcription of subgenomic RNAs.^{4,5}

Infectious bronchitis virus (IBV) is a Group 3 coronavirus, members of which infect avian species. IBV produces 6 subgenomic RNAs. Even though the TRS is known, importance of TRS itself or adjacent sequences have not been addressed. In this study, using an infectious cDNA clone of IBV and targeted mutagenesis, we show that the sequences surrounding the IBV TRS contribute to subgenomic RNA regulation.

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2. MATERIALS AND METHODS

Site-directed mutations near TRS3 were introduced using PCR-based site-directed mutagenesis, and recombinant IBV containing these mutations were constructed using an infectious cDNA clone of IBV as described previously.⁶ The subgenomic RNA profile of IBV and recombinant IBVs were compared by Northern blot analysis. Total cellular RNAs were extracted from infected Vero cells and electrophoresed on 1% denaturing agarose gel containing 2.2M formaldehyde. A ³²P-labeled anti-sense IBV 3' UTR probe was used to detect the subgenomic RNAs of IBV.

3. RESULTS

3.1. Four Nucleotide Changes Downstream of the RNA3 TRS Attenuates RNA Transcription

A canonical dilysine endoplasmic reticulum (ER) retrieval signal was previously identified in near the C-terminus of the IBV S protein.⁷ To address the significance of this trafficking signal to IBV infection, we used an infectious cDNA clone of IBV.⁶ We produced a recombinant virus with a mutation in the ER retrieval signal of the S protein, which is encoded by subgenomic RNA2 (IBV-S2A, Fig. 1). IBV-S2A gave an interesting phenotype: not only did it produce less total virus and form bigger plaques (as expected), but there was also significantly impaired virus release from infected cells (which was unexpected). Further analysis of subgenomic RNA by Northern blot showed that cells infected with IBV-S2A had significantly less RNA3 transcript than those infected with the parental IBV (Fig. 2B). The reduction in RNA3 levels was detected at both early and late times post-infection, and was mirrored by decreased E protein expression, which is one of the ORFs encoded by RNA3. Interestingly, the ER retrieval signal of IBV S is located 10 nucleotides downstream of RNA3 core TRS (Fig. 1).

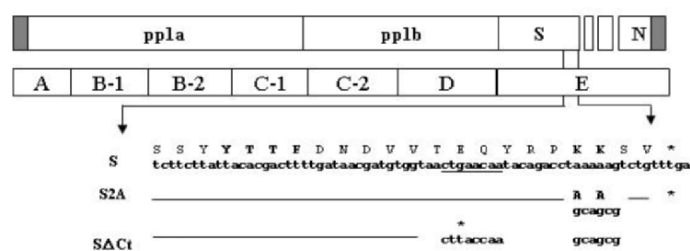


Figure 1. Schematic of recombinant IBV construction and introduced mutations. Canonical dilysine ER retrieval signal in the IBV S C-terminus (bold) was replaced with two alanines (IBV-S2A). A recombinant virus with a premature stop codon upstream of the ER retrieval signal but containing the alanine codons was rescued serendipitously (SΔCt). The TRS of RNA3 is underlined. Asterisks indicate stop codons. Lines represent nucleotide and amino acid sequences that are identical.

3.2. Two Different Contexts of Core TRS and Transcription Regulation

There are not many complete genomic sequences of IBV available. However, based on the available sequence data, the TRS of IBV is well conserved among different strains. Interestingly, unlike other coronaviruses, the IBV genome has two different TRS core sequences (Fig. 2A). The major one is CTTAACAA, which is used as the core TRS for the leader and subgenomic RNA4, 5 and 6. The minor TRS is CTGAACAA, which acts as the core TRS for subgenomic RNA2 and 3. Except for the genomic RNA, RNAs transcribed under the control of the major TRS are produced at higher levels than those under control of the minor TRS (RNA2 and 3, Fig. 2B). One of the mutants rescued while studying the effects of the IBV S ER retrieval signal on IBV infection had a nucleotide change in the TRS. This mutation encodes a premature stop codon in IBV S protein, resulting in a truncated protein lacking the ER retrieval sequence (IBV-SΔCt, Fig. 1). The mutation in this virus also changed the RNA3 TRS from CTGAACAA to CTTAACAA (resulting in a perfect match with the leader TRS). However, Northern blot analysis of RNA from cells infected with this mutant showed that the change to the major TRS did not overcome the RNA3 transcription attenuation caused by the downstream nucleotide changes (data not shown).

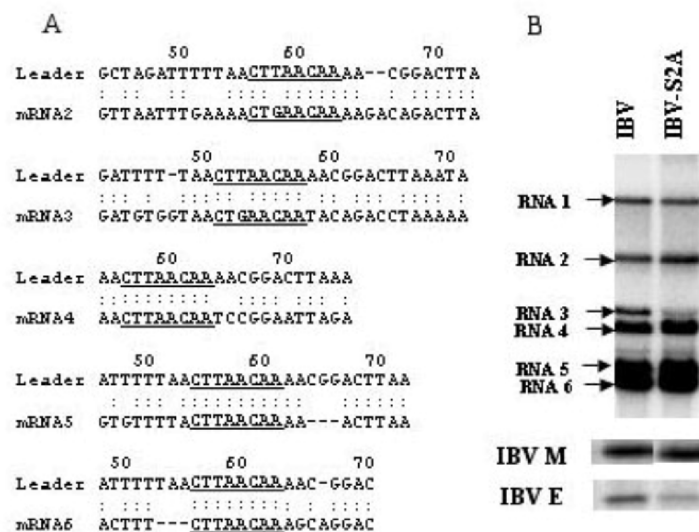


Figure 2. (A) Sequence alignment of each subgenomic RNA TRS with the 3' end of the leader core TRS. The TRS of RNAs 4, 5, and 6 shows perfect sequence homology with the leader core TRS, by contrast with the TRS of RNAs 2 and 3. (B) Vero cells were infected with IBV or IBV-S2A and total RNAs were extracted from the infected cells and subjected to Northern blot analysis using a ³²P random primed anti-sense IBV 3' UTR RNA as a probe (upper panel). Immunoblot for IBV M and IBV E proteins was also performed from the same samples (lower panel). Four nucleotide changes downstream of the RNA3 TRS result in less RNA3 transcript compared with the RNA3 transcript levels in cells infected with the parental IBV. Reduced RNA3 transcript was confirmed by lower expression level of E protein.

4. DISCUSSION

The genome of IBV is smaller than other coronaviruses (27.6 vs. 29 to 31 kb). However, all of the necessary information is accommodated by overlapping most of the ORFs. Except for RNA1 and RNA5, the TRS for each ORF contains codons that conserve not only the TRS but also the amino acid sequence of the protein encoded on the upstream ORF. For example, IBV S protein (encoded by RNA2) contains targeting signals on its cytoplasmic tail. One of these signals is an ER retrieval signal, which is potentially important for viral assembly. Interestingly, this ER retrieval signal is 10 nucleotides downstream from the RNA3 TRS. Disruption of the ER retrieval signal by 4 nucleotide changes also attenuated transcription of the downstream subgenomic RNA (RNA3) with a corresponding decrease in E protein expression. This is the first study showing that for IBV, both the TRS and neighboring sequences affect subgenomic RNA transcription.

When we isolated the recombinant virus that lacked the ER retrieval signal, we identified a mutant that had a premature stop codon upstream of the ER retrieval signal. This mutation created a perfect match between the leader TRS and the TRS for RNA3. However, the downstream nucleotide changes (in the ER retrieval sequence) remained and perhaps explain why this mutation did not rescue the RNA3 level to normal. Interestingly, the IBV field isolate Mass 41 has the same premature stop codon in the S gene that resides in the RNA3 TRS core sequence of IBV- Δ Ct, rendering a perfect match in body and leader TRS for RNA3. However, this virus conserved the nucleotide sequence in the ER retrieval signal. It will be interesting to see if the Mass 41 strain of IBV produces a normal or even higher level of subgenomic RNA3 compared with other strains of IBV.

5. ACKNOWLEDGMENTS

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