Characterization of an Arterivirus Defective Interfering RNA

Replication and homologous recombination

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1. INTRODUCTION

Recently, we have described the generation of DI-b, a natural equine arteritis virus (EAV) defective interfering (DI) RNA of 5.6 kb, and we have reported the construction of pEDI, a full-length cDNA copy of EAV DI-b RNA from which replication-competent RNA can be transcribed *in vitro* (Molenkamp *et al.*, 2000a, Molenkamp *et al.*, 2000b). EDI RNA consists of three noncontiguous parts of the EAV genome fused in frame with respect to the replicase gene (Fig. 1). As a result the EDI RNA contains a truncated replicase open reading frame (ORF), which we will refer to as EDI-ORF. The importance of such a translation unit in coronavirus DI RNA propagation has been shown by a number of researchers (de Groot *et al.*, 1992; van der Most *et al.*, 1995; Liao and Lai, 1995).

In this study we have analyzed the importance of the EDI-ORF in EDI RNA replication. The EDI-ORF was disrupted at different positions by the introduction of frameshift mutations. These were found either to block DI RNA replication completely or to be removed during virus passaging, probably due to homologous recombination with the helper virus genome. Using recombination assays based on EDI RNA and full-length EAV genomes containing specific mutations, the rate of homologous RNA recombination in the 3'- and 5'-proximal regions of the EAV genome was

studied. Remarkably, the recombination frequency in the 5'-proximal region was found to be approximately 100-fold lower than that in the 3'-proximal part of the genome.

Figure 1. Schematic representation of the EAV genome and EDI RNA. Leader and body TRSs (see below) are depicted by arrowheads. The three noncontiguous parts of the EAV genome of which EDI consists are indicated

2. RESULTS

In view of the presence of a large ORF in many natural nidovirus DI RNAs, we have investigated the significance of the EDI-ORF in EAV DI RNA propagation. To this end, we engineered a set of pEDI derivatives containing C-terminally truncated EDI-ORFs of different sizes. By using a number of convenient restriction sites, we introduced frameshift mutations into the EDI-ORF. Furthermore, in construct pEDI-UAC, the EDI-ORF

Table 1. Overview of EDI-ORF frameshift mutants.

Construct	Restriction site	Position (nt) ^a	Position stop codon (nt) ^a	Length ORF
pEDI			2576	784
pEDI-UAC	N.A.	224	224	0
pEDI-127	<i>Mlu</i> I	589	605	127
pEDI-215	<i>Sst</i> I	857	869	215
pEDI-404	<i>Nco</i> I	1391	1436	404
pEDI-550	<i>Sal</i> I	1821	1874	550
pEDI-616	<i>Bam</i> HI	1975	2072	616

translation initiation codon was mutated. An overview of the EDI-ORF mutants is presented in Table 1. In vitro-transcribed RNA of the EDI-ORF mutants was transfected into EAV-infected baby hamster kidney (BHK-21) cells, and standard (undiluted) virus passaging experiments (Molenkamp *et al.*, 2000a) were performed. Intracellular RNA was isolated after P2 and analyzed by denaturing agarose gel electrophoresis and hybridization (Fig. 2). The positive control, EDI RNA containing the full-length EDI-ORF (784 aa), was replicated and passaged efficiently. Remarkably, RNA derived from the EDI-UAC translation initiation codon mutant could not be detected in P2 RNA samples. Likewise, the EDI-ORF mutants containing a relatively short reading frame (EDI-127 and EDI-215) could not be rescued. These results suggest that either translation of the 5'-terminal half of EDI-ORF or the N-terminal part of the EDI-protein is required for passaging of this EAV DI RNA.

Figure 2. Analysis of the replication of EDI-ORF frameshift mutants. RNA from pEDI and EDI-ORF frameshift mutants was transfected into EAV-infected BHK-21 cells. Virus was harvested at 16 h posttransfection and passaged twice. P2 RNA was isolated at 12 h p.i. and subjected to gel electrophoresis and hybridization with an oligonucleotide recognizing the 3' ends of all viral mRNAs. The mock lane represents cells that were EAV infected but not transfected.

To analyze whether escape mutants containing a restored EDI-ORF were generated, intracellular P2 RNA of all EDI-ORF mutants was analyzed. The

region corresponding to the EDI-ORF was amplified by using an EDI RNA-specific RT-PCR. PCR products were analyzed on agarose gel (data not shown) and the region containing the frameshift mutation was sequenced for each of the mutants that yielded a product. To our surprise, in all EDI-ORF frameshift mutants that were rescued efficiently (EDI-404, EDI-550, and EDI-616), the restriction site used to generate the frameshift mutation had been repaired and the flanking sequences in this region were completely identical to the wild-type (wt) EAV sequence. This strongly suggested that these mutants had undergone homologous recombination with the helper virus genome.

The results obtained with our EDI-ORF mutants strongly suggested that the rates of recombination may vary in different regions of the genome. To characterize this phenomenon in more detail, we investigated the relative rates of RNA recombination in the 3'- and 5'-proximal regions of the EAV genome. We designed recombination assays in which we made use of two previously described non-infectious but replication-competent full-length cDNA clones, mutants L3 and B3 (van Marle *et al.*, 1999). In these constructs, either the leader transcription-regulating sequence (TRS) (L3 mutant) or the RNA7 body TRS (B3 mutant) has been changed from 5'-UCAAC-3' to 5'-UGAAG-3'. In mutant L3 sg mRNA synthesis is completely abolished, whereas mutant B3 does not generate sg mRNA7. Thus, these mutants could serve as a potential partner in RNA recombination, which would yield an infectious EAV genome when the region containing the L3 or B3 TRS mutation would be exchanged for the corresponding region of an EDI RNA.

To analyze the rate of recombination in the 3'-proximal region of the EAV genome, mutant B3 was cotransfected with EDIC2-4150 RNA (Molenkamp *et al.*, 2000a). This EDI derivative contained the CAT reporter gene at the position normally occupied by ORF2b. In addition, EDIC2-4150 contains a deletion ranging from the 3' end of ORF3 (EAV nt 10723) to the 3' end of ORF5 (EAV nt 11636). EDIC2-4150 contained the wt RNA7 body TRS, and hence a single recombination with the B3 RNA in the 616-nt region between the 3' border of the deletion and the RNA7 TRS of EDIC2-4150 could yield a full-length, infectious EAV genome. Likewise, the rate of recombination in the 5'-proximal region of the genome was analyzed by cotransfection of the L3 mutant and EDIC2-4150. In this case, a single recombination event occurring in the 846-nt region between the leader TRS and the fusion of the first and second EDI segments of EDIC2-4150 (fusion site A) could generate a full-length, infectious RNA molecule. CAT expression was used to determine the number of double-transfected cells in an immunofluorescence assay (+/- 10% for both transfections). Medium from double-transfected cells was harvested at 12 h and the presence of infectious virus particles was determined by plaque assays (Fig. 3). Surprisingly, medium harvested from cotransfections of mutant B3 and

EDIC2-4150 (Fig. 3) contained substantially more recombinant virus particles than medium harvested from cotransfections of mutant L3 and EDIC2-4150 (Fig. 3). Only when a large amount of a 24-h harvest of the L3/EDIC2-4150 double transfection was used, a single plaque was observed (Fig. 3). Upon titration of the 24-h harvests from single transfections of mutant B3, mutant L3, or EDIC2-4150, no plaques were detected (Fig. 3), indicating that reversion of the L3 and B3 TRS mutations did not occur during the 24-h incubation period. We estimated that recombination in the 5'-proximal region of the genome was at least 100-fold less efficient than recombination in the 3'-terminal region.

Figure 3. Titration of recombinant viruses. Medium from cotransfections of the B3 mutant (A) or L3 mutant (B) and EDIC2-4150 was harvested at 12 h posttransfection and titrated in plaque assays. Dilutions ranged from undiluted (upper left well) to 10^{-5} (lower right well). (C) Undiluted medium from a cotransfection of mutant L3 and EDIC2-4150, harvested at 24 h posttransfection, was used for a large-scale plaque assay. The single plaque observed is indicated with an arrow. (D) Control experiment. Medium isolated from single transfections of either the B3 mutant, the L3 mutant, or EDIC-4150 was harvested at 24 h posttransfection and used in a plaque assay. No plaques were detected.

3. DISCUSSION

In this paper we demonstrate the importance of the EDI-ORF for the propagation of EDI RNA. As proposed by de Groot *et al.* (1992) and supported by van der Most *et al.* (1995), translation of the ORF in coronavirus DI RNAs, which often spans almost the entire DI genome, could enhance DI RNA stability. Consequently, the fitness of DI RNAs with an interrupted or truncated ORF might be reduced. In the case of EDI, the ORF covers only half of the RNA molecule (Molenkamp *et al.*, 2000a). However, it is interesting to note that in the region downstream of the truncated replicase ORF, in contrast to other nidovirus DI RNAs, EDI has retained the genomic 3'-terminal region that includes the *cis*-acting sequences required for the synthesis of all sg mRNAs. Therefore, we speculate that the nontranslated 3'-terminal part of the EDI genome might be stabilized by sg mRNA transcription rather than by translation. An alternative explanation for the requirement for a DI ORF is that one or more of the proteins it encodes is needed in *cis* for the replication of the DI RNA.

In this study, we compared the relative rates of recombination in the 5'- and 3'-proximal regions of an EAV DI RNA and mutant full-length genomes. In addition to recombinants, pseudotype virions could have been produced, containing both the DI RNA and the mutant genome, which might complement each other at the level of protein expression. However, our plaque assays and the analysis of the viral RNA isolated from individual plaques revealed that pseudotype virions were not generated (data not shown). Interestingly, our data showed that the relative rates of recombination in different regions of the EAV genome can vary. The relative recombination frequency in the 5'-proximal region of the genome was found to be approximately 100-fold lower than that in the 3'-terminal region. The high rate of recombination in the 3' end of the genome might be a direct result of the discontinuous nature of nidovirus sg RNA transcription. It has been proposed that minus-strand RNA synthesis is attenuated at body TRSs, a step which should be followed by base pairing with the leader TRS and reinitiation of minus-strand synthesis to add the antileader sequence (Konings *et al.*, 1988; Sawicki and Sawicki, 1995; van Marle *et al.*, 1999; van Marle *et al.*, 1995). In a similar manner, attenuation of minus strand synthesis might facilitate RNA recombination. The polymerase complex could reinitiate transcription of the nascent minus-strand at the same body TRS of a different template, and thus in our case continue to transcribe a recombinant full-length genome after initiating minus strand RNA synthesis from an EDI template. At the 5' end of the EAV genome, minus-strand synthesis might be attenuated much less frequently, which could explain the much lower rate of recombination in this region.

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