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A STRATEGY FOR THE GENERATION OF INFECTIOUS RNAs AND AUTONOMOUSLY REPLICATING RNAs BASED ON THE HCV 229E GENOME

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1. ABSTRACT

A strategy to generate in vitro transcripts representing infectious RNAs and autonomously replicating RNAs based on the HCV 229E genome is presented. PCR-DNAs were ligated *in vitro,* resulting in 27 kbp and 22 kbp ligation products. These DNAs can now be transcribed *in vitro* and the RNAs tested for infectivity and their ability to replicate.

2. INTRODUCTION

The generation of infectious transcripts from cloned DNAs equivalent to coronaviral genomes has been hampered by a variety of problems. For example, vectors carrying coronaviral polymerase sequences are often unstable upon propagation in bacteria. Also, the size of the coronaviral genome prevents conventional cloning techniques in the common plasmid or bacteriophage vectors.

We have developed a system that allows for the generation of exceptionally long HCV 229E specific RT-PCR derived DNAs, starting with polyA-RNA isolated from HCV 229E infected cells. These DNAs are ligated in vitro to generate DNAs representing replicationally active RNA molecules.

Coronaviruses and Arteriviruses, edited by Enjuanes *et al.*
Plenum Press, New York, 1998

3. METHODS AND RESULTS

3.1. The RT-PCR Derived DNAs

We are able to generate RT-PCR derived DNA molecules that together represent either a full length DNA copy of the HCV 229E genome or a DNA encoding the 5- and 3′-cis acting signals necessary for replication and transcription combined with the coronaviral RNA polymerase gene i.e. a DNA copy of an autonomously replicating HCV 229E RNA.

The DNA fragments (AI, AII and AIII or BI, BII and BIII, Fig. 1) were obtained using primers containing coronavirus specific sequences and either a T7 RNA polymerase

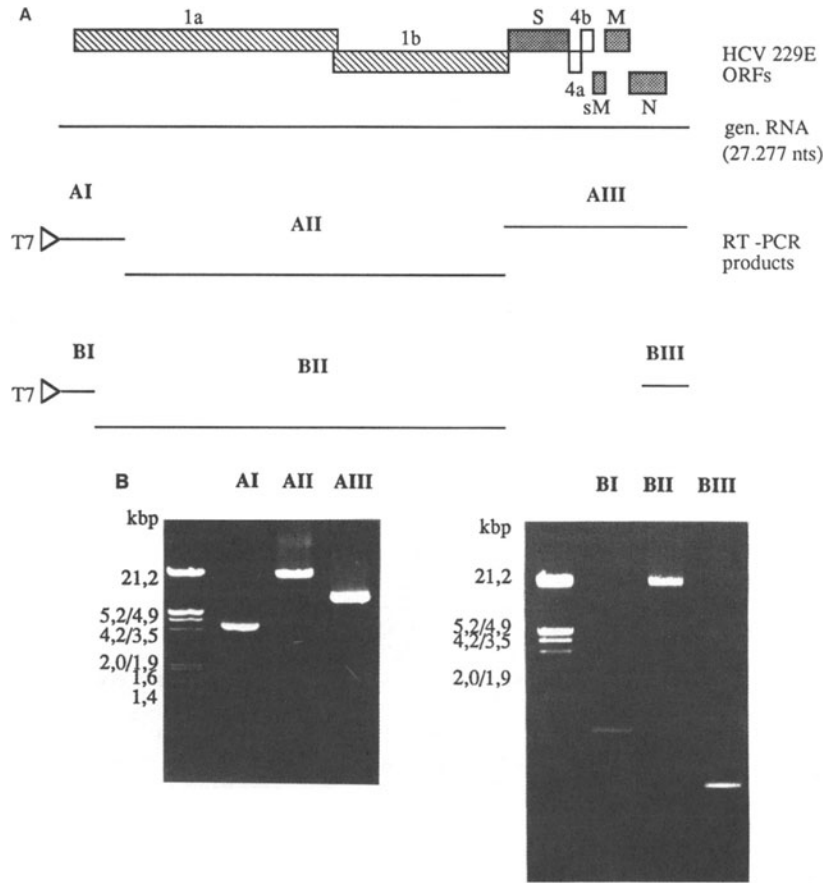

Figure 1. RT-PCR DNAs representing either (A) the genome of HCV 229E or (B) an autonomously replicating RNA. In the upper scheme, the position of the DNAs, relative to the HCV 229E genome, is shown. The DNAs were separated on 0.7% agarose gels to verify the correct length and homogeneity of the RT-PCR products.

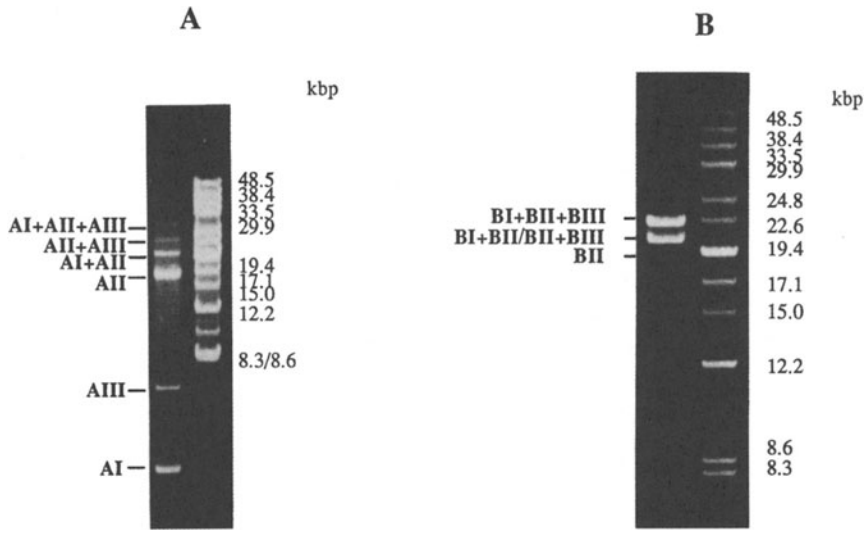

Figure 2. Ligation reaction products representing (A) the genome of HCV 229E (AI+AII+AIII) or (B) an autonomously replicating RNA (BI+BII+BIII). The ligated DNAs have been separated on a 0.8% agarose gel using the pulsed-field electrophoresis. The RT-PCR DNAs, intermediate reaction products and the full-length reaction products are shown.

promotor (fragments AI and BI, upstream primer), specific restriction enzyme recognition sites (all fragments) and marker mutations that allow for the identification of recombinant genomic RNA(all fragments).

3.2. In Vitro Ligation

The RT -PCR DNAs were treated with restriction enzymes to generate compatible ends. Subsequently the DNAs were purified and ligated in vitro using T4 DNA ligase. The reaction products were separated by PFGE (Fig. 2). Although the amounts of the full length reaction products are low, we could clearly detect the ligation products AI+AII+AIII and BI+BII+BIII. It is obvious that complete digestion of the RT-PCR DNA ends is the most critical parameter for complete ligation. In the future we will use this in vitro ligation technology to ligate quantitative amounts of PCR DNAs that can be subsequently used for in vitro transcription.

3.3. In Vitro Transcription

We have also tested the ability of T7 RNA polymerase to generate long HCV 229E-specific transcripts in vitro. A series of RT-PCR DNAs, representing the first 2 to 10 kb of the HCV 229E genome, with a T7 RNA polymerase promotor at the 5´-end has been synthesized and transcribed in vitro using T7 RNA polymerase.

Fig. 3 shows that T7 RNA polymerase is able to transcribe efficiently RNA using RT-PCR DNAs as substrates (approx. 3–10 μgRNA/μg DNA). Allthough the efficiency de-

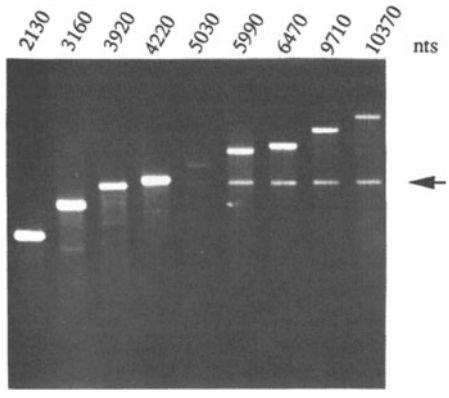

Figure 3. RT-PCR DNAs containing a T7 RNA polymerase promotor at the 5′end were transcribed in vitro using T7 RNA polymerase. After transcription, the template DNA was digested with RQI-DNase and the RNA was isolated by $LiCl_2$ precipitation. The RNAs (0.5 µg) were then separated by electrophoresis in a 1% agarose gel containing 0.1% SDS. The arrow indicates the RNA species derived by premature termination of the T7 RNA polymerase.

creases with the length of the DNA template sufficient amounts of 10 kb RNA molecules are synthesized. In this experiment, we have also identified a T7 RNA polymerase termination signal encoded in the RT-PCR DNA, located between nt 3.900 and 4.200 of the HCV 229E genome. A possible candidate is the heptanucleotide sequence ATCTGTT starting at position 4039. This heptanucleotide has been described recently to be a T7 RNA polymerase termination signal in vitro (Hartvig and Christiansen, 1996).

4. DISCUSSION

We intend to use the above strategy to produce quantitative amounts of RNAs equivalent to both the HCV 229E genome [containing marker mutations] and an autonomously replicating RNA. For this purpose, we will have to optimize at least two conditions: (i) in order to increase the amount of the full length in vitro ligation products, we will have to control the extent of endonucleolytic digestion of the RT-PCR DNAs and (ii) we will have to compare bacteriophage T7, T3, and SP6 RNA polymerases for their ability to synthesize long transcripts in vitro. It will also be necessary to avoid the generation of additional RNA species due to cryptic RNA polymerase termination signals. These RNAs will then be transfected into eukaryotic cells and screened for their ability to replicate and, eventually, to initiate an infectious cycle. We hope that the ability to produce full length infectious transcripts will greatly facilitate both molecular and pathogenesis studies on coronaviruses. In the long term, autonomously replicating coronavirus RNAs could also be used to establish a system for the expression of foreign genes in eukaryotic cells.

REFERENCES

Hartvig, L., and Christiansen, J., 1996, Intrinsic termination of T7 RNA polymerase mediated either by RNA or DNA, *EMBO J.* **15**:4767–4774.