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REPLICATION AND TRANSCRIPTION OF HCV 229E REPLICONS

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1. ABSTRACT

Replicons based upon the human coronavirus 229E (HCV 229E) genome were transfected into HCV 229E infected cells. We demonstrate that a synthetic RNA comprised of 646 nucloetides from the 5' end and 1465 nucloetides from the 3' end of the HCV 229E genome is replication competent. We conclude that the cis-acting elements necessary for replication are located in these 5' and 3' genomic regions. Furthermore, we inserted the intergenic region of the HCV 229E nucleocapsid protein gene into this basic construct and were able to demonstrate the transcription of "subgenomic" RNAs

2. INTRODUCTION

The study of coronavirus replication has been greatly facilitated by the use of defective interfering (DI) RNAs that can replicate in coronavirus infected cells (Makino and Lai, 1989; Van der Most et al., 1991). The analysis of several different DI RNAs has revealed that cis-acting elements required for replication are located at the 5' and 3' end of the coronavirus genome. Furthermore, the insertion of an intergenic sequence into these DI RNAs leads to the synthesis of "subgenomic" RNA transcripts (Makino et al., 1991). In order to establish a similar system to study HCV 229E replication, we have constructed an HCV 229E replicon containing 5' and 3' sequences of the HCV 229E genome behind a T7 RNA polymerase promoter. Furthermore, we have inserted the intergenic sequence of the HCV 229E nucleocapsid protein gene at different positions within this construct and tested for replication and transcription. The potential use of this repli-

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cation and transcription assay in combination with a recombinant expressed HCV 229E RNA polymerase is discussed.

3. METHODS

3.1. DNA Construction

A PCR fragment was generated using DNA of the plasmid T35D5 (Herold et al., 1993) as template and the primers 35D5(up) (5'-ATCAGGGAATTCTAATACGACTCAC TATAGGGACTTAAGTACCTTATCTATCTACAGATAGAAAAGTTGCTTTTTA-3') and 35D5(down) (5'-ATTTGCGGGTACCCTCTGGTCAGTGTATGTCACATT-3'). The primer 35D5(up) contains an EcoRI restriction site followed by the T7 RNA polymerase promoter, three G bases and 44 nucleotides corresponding to the 5' end of the HCV 229E genome. The primer 35D5(down) contains a KpnI restriction site preceded by 21 nucleotides complementary to the nucloetides 626 - 646 of the HCV 229E genome. A second PCR fragment was generated using the plasmid pBH1 as template and the primers BH1(up) (5'-CCA-CAACGTGGTCGTCAGGGT-3') and BH1(down) (5'-ACTAGTGGATCCCCCCGA-CATCGATTT-3'). The plasmid pBH1 contains the HCV 229E genomic sequence from nt. 23868 to the 3' end of the genome (Herold, unpublished). The primer BH1(up) corresponds to the nucloetides 25719–25739 of the HCV 229E RNA sequence and the primer BH1(down) contains a BamHI restriction site preceded by 12 nucleotides that correspond to the sequence downstream of the poly(A) stretch in the construct pBH1. Both PCR fragments were cleaved with KpnI and ligated. The ligation product was cleaved with EcoRI and BamHI and inserted into the EcoRI and BamHI cleaved vector pBR322. The resulting construct is designated as pRep1.1. In order to insert an intergenic sequence, two annealed oligonucleotides with KpnI overhangs were cloned into the KpnI site of pRep1.1. The inserted sequence corresponds to the nucleotides 25656–25684 of the HCV 229E sequence (including the intergenic sequence of the HCV 229E nucleocapsid protein gene) flanked by 6 nucleotides upstream and 30 nucleotides downstream that were introduced for further cloning procedures. The resulting construct is designated as pRep6.1. To obtain the constructs pRep6.2, pRep6.3 and pRep6.4, we inserted the same sequence into pRep1.1 using the Bsu36I, BstXI and PacI sites, respectively. The intergenic sequence of the constructs pRep6.1, pRep6.2, pRep6.3 and pRep6.4 is located 1495, 1030, 802 and 277 nucleotides, respectively, upstream of the poly(A) stretch representing the 3' end of the HCV 229E genomic sequence.

3.2. RNA Replication and Transcription Assay

In vitro transcription with T7 RNA polymerase was done with BamHI-linearised plasmid DNA templates using the MEGAscript-Kit (Ambion, Germany). 1×10^5 HeLa cells were infected with HCV 229E (m.o.i. 10) and incubated at 33°C. At 1h post infection, 5 µg in vitro synthesised RNA was transfected using 12.5 µl Lipofectin (Life Technologies, Germany) according to the manufacturer's instructions. At 30 min post transfection the cells were washed twice with medium and incubated for further 16 h at 33°C. Poly(A) RNA was prepared using oligo $(dT)_{25}$ dynabeads (Dynal) according to the manufacturer's instructions and electrophoresed on 2.2 M formaldehyde-1% agarose gels. The gels were dried and hybridised with a ^{32}P-(5'-end)-labelled oligonucleotide, as described previously (Meinkoth and Wahl, 1984).

4. RESULTS

4.1. The Structure of HCV 229E Replicons

Previous studies on coronavirus replication have shown that RNAs containing 5' and 3' genomic sequences can replicate in coronavirus infected cells. However, there has been no analysis of the RNA sequences that are necessary for replication in HCV 229E infected cells. To identify the cis-acting sequences that are sufficient for the replication of RNAs by the HCV 229E RNA polymerase, we cloned 646 nucleotides from the 5' end and 1465 nucleotides from the 3' end of the HCV 229E genome behind a T7 RNA polymerase promoter. To allow efficient in vitro transcription with T7 RNA polymerase, we inserted three additional G bases between the T7 promoter and the 5' end of the HCV 229E sequence. A BamHI restriction site, 11 nucleotides downstream of the poly(A) sequence of the HCV 229E 3' end, was used to linearise plasmid DNA prior to in vitro transcription. The structure of this construct, designated as pRep1.1, is shown in Figure 1. In order to analyse transcription, we inserted the intergenic sequence of the HCV 229E nucleocapsid protein gene at different positions in the pRep1.1 sequence as illustrated in Figure 1. The resulting constructs are designated as pRep 6.1, 6.2, 6.3 and 6.4.

The structure of the construct pRep 1.1 relative to the HCV 229E genome is shown. The position of the insertion of the intergenic sequence in the constructs pRep 6.1, 6.2, 6.3 and 6.4 are indicated.

4.2. Replication and Transcription

To study replication and transcription by the HCV 229E RNA polymerase, we synthesised in vitro transcripts of the HCV 229E replicons using T7 RNA polymerase. Then, 5 µg of these synthetic RNAs were transfected into 1×10^5 HeLa cells that had been previously infected with HCV 229E. After 16 h incubation at 33 °C, the cells were lysed and the poly(A) RNA was isolated. The RNA samples were separated by gel electrophoresis

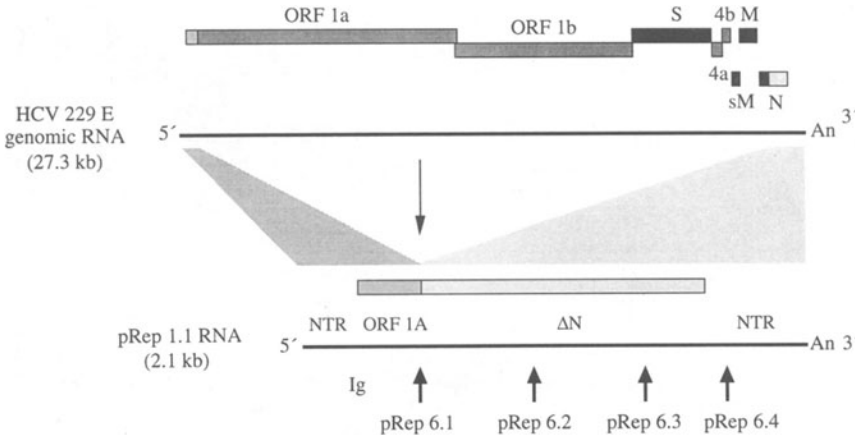

Figure 1. Schematic representation of HCV 229E replicons.

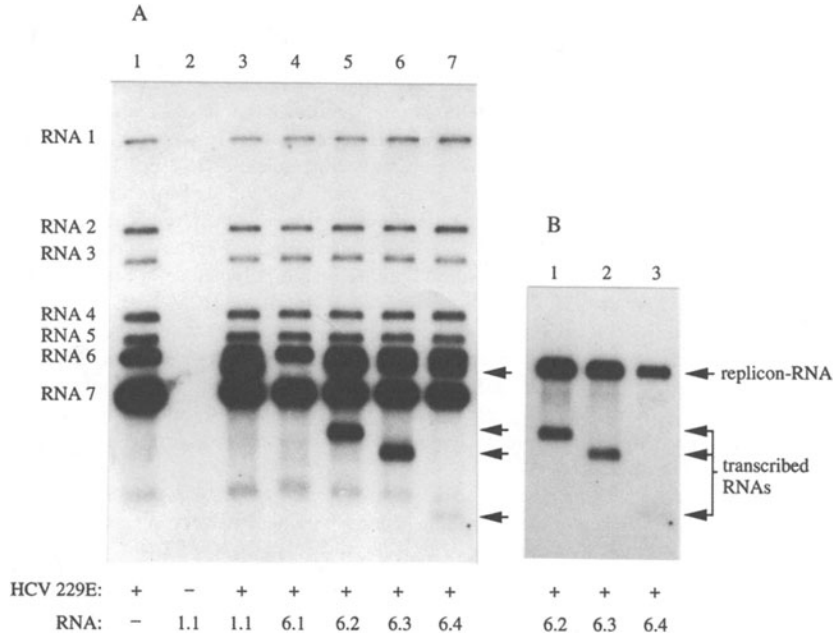

Figure 2. Replication and transcription assay.

and hybridised with a HCV 229E nucleocapsid protein gene specific ^{32}P labelled oligonucleotide. The result of this analysis is shown in Figure 2a.

Lane 1 shows poly(A) RNA from HCV 229E infected HeLa cells. The HCV 229E genomic RNA and six subgenomic mRNA can be detected. Lane 2 shows that transfected RNA derived from pRep1.1 cannot be detected in unifected cells. In contrast, as is shown in lane 3, RNA derived from pRep1.1 is replicated in HCV 229E-infected cells. Lanes 5, 6 and 7 show that this is also true for RNA derived from pRep6.2, pRep6.3 and pRep6.4. RNA derived from pREp6.1 did not replicate in infected cells (lane 4) and the reason for this difference remains to be determined. Importantly, we were also able to detect "subgenomic" RNAs transcribed from the RNA derived from pRep6.2, pRep6.3 and pRep6.4. Depending on the position of the intergenic sequence in the replicon construct, "subgenomic" RNAs of 1.0 kb, 0.8 kb and 0.3 kb, respectively, were detected (lanes 5, 6 and 7).

Poly(A) RNAs were separated by electrophoresis. Viral RNAs were hybridised with a) an HCV 229E nucleocapsid gene-specific or b) a replicon-specific, 5'-end-labelled oligonucleotide. Viral and replicon specific RNAs are indicated.

To further confirm the identity of the replicated and transcribed RNAs, we did a second hybridisation analysis. In this case, the nucleocapsid protein gene specific ^{32}P labelled oligonucleotide was replaced by a ^{32}P labelled oligonucleotide specific for the insertion of the HCV 229E intergenic sequence. The result is shown in Figure 2b. As expected, the viral RNAs can no longer be detected, whereas the replicons (with a size of approximately 2.1 kb) are clearly visible. Furthermore, the detection of the "subgenomic RNAs (1.0, 0.8

and 0.3 kb) demonstrate transcription from these replicating RNAs. Again the sizes of the transcribed RNAs correlate to the position of the intergenic sequence in the replicon construct.

5. DISCUSSION

In this study, we have established an assay to detect HCV 229E RNA polymerase activity using HCV 229E derived replicating RNA. We have tested several synthetic RNAs comprised of 5' and 3' genomic sequences for their ability to replicate in HCV 229E infected cells. This analysis revealed that 646 nts from the 5' end and 1465 nts from the 3' end of the HCV 229E genome are sufficient for replication. We therefore conclude that all cis-acting sequences necessary for replication are located in these 5' and 3' genomic regions. Furthermore, we have demonstrated transcription of "subgenomic" RNAs from replicating RNA, under the control of an intergenic region.

The use of assays similar to those reported here have greatly enhanced our understanding of, in particular, cis-acting elements and their role in coronaviral RNA replication and transcription. However, the structure-function relationships of the coronavirus RNA polymerase still remain, by and large, an enigma. The RNA polymerase gene is comprised of two overlapping ORFs with a length of about 20 kb. The primary polymerase translation products are extensively processed into smaller polypeptides and it is not yet known which polymerase gene products participate in the many and complex functions of the replication/transcription process. To approach such questions, it will be necessary to clone a functional RNA polymerase gene and establish a system that allows for the use of reverse genetics. One possibility is to express the RNA polymerase in eucaryotic cells using recombinant vaccinia viruses. The replication/transcription assay presented in this study represents an important step towards achieving this goal.

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