Characterization of a Nucleic Acid Probe for the Diagnosis of Human Coronavirus 229E Infections

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A cDNA copy of the HCV229E nucleocapsid protein gene was isolated and characterized. Sequence analysis predicts a nucleocapsid polypeptide of 389 amino acids with a molecular weight (mol. wt.) of 43,450. Single strand RNA probes derived from the cDNA copy hybridize specifically to HCV229E RNA and approximately 50 pg of intracellular viral RNA can be readily detected. The application of nucleic acid hybridization as a routine procedure for the diagnosis of HCV229E infection is discussed.

KEY WORDS: N gene sequence, hybridization analysis, coronavirus

INTRODUCTION

Human coronaviruses (HCVs) are causative agents of respiratory illness in man. In healthy adults they are associated with common colds of mild to moderate severity, usually of 6-8 days duration. The typical symptoms are nasal catarrh and sore throat, although headache, fever, diarrhea, and other symptoms are occasionally reported. Epidemiological data indicate that worldwide HCVs account for 5-35% of all upper respiratory tract infections. HCV infection in children may also lead to lower respiratory illness, including bronchitis and pneumonia. [For a review of the biology and pathogenesis of HCVs see Hierholzer and Tannock, 1988.]

HCVs can be divided into two major antigenic groups represented by HCV229E and HCVOC43 [Macnaughton et al., 1981; Pedersen et al., 1978]. The HCV229E virion is comprised of a positive strand RNA genome, which if HCV is similar to other coronaviruses is about 30 kilobases (kb) in length; a lipid envelope; and three major proteins: the nucleocapsid protein, N (mol. wt. 50,000); the membrane glycoprotein, M (mol. wt. 21,000-25,000); and the spike glycoprotein, S (mol. wt. 186,000) [Kemp et al., 1984; Macnaughton and Madge, 1978; Schmidt and Kenny, 1982]. Viruses of the OC43 group have an additional surface glycoprotein, the haemagglutinin-esterase, HE (mol. wt. 65 × 10^3) [Hogue and Brian, 1986].

The replication of HCV229E involves the synthesis of a 3' co-terminal set of six subgenomic RNAs [Weiss and Leibowitz, 1981]. It is assumed that these RNAs are synthesized in the cytoplasm of infected cells by a process of leader-primed discontinuous transcription, as has been described for the murine hepatitis virus, MHV [Baric et al., 1985; Makino et al., 1986a; Shieh et al., 1987]. Recently, the HCV229E genes encoding the nucleocapsid, membrane, and spike proteins have been cloned and sequenced and their order on the genome has been determined as 5' S-M-N 3' [Raabe and Siddell, 1989a; Raabe et al., 1990; Schreiber et al., 1989]. Raabe et al. [1990] have proposed that the subgenomic RNAs 2, 6, 7 function as the mRNAs for the S, M, and N proteins, respectively.

Basically, three approaches to the diagnosis of HCV infections have been used. Firstly, there is the isolation and identification of HCVs in cell and organ culture. However, this approach is time consuming and requires considerable expertise. Only HCV229E-like viruses can be directly isolated in cell culture and other HCVs have to be isolated in organ culture and, if possible, adapted to cell monolayers [see Hierholzer and Tannock, 1988].

The second approach is serological. Almost all the standard tests, including serum neutralization, complement fixation, and haemagglutinin inhibition (for OC43-like virus) have been used, but they are relatively insensitive and are not widely applied. More recently, enzyme-linked immunoassays have been developed [Kraaijeveld et al., 1980; Macnaughton et al., 1981, 1982] and at the present time they provide the most reliable epidemiological information on HCV infections. The major drawback is that paired sera are required and due to the ubiquity of HCV, and the frequency of reinfection, the differences in antibody titers between acute and convalescent sera are rarely dramatic.

The third approach to HCV diagnosis involves the direct detection of viral antigens in nasal and pharyn-
geal swabs or aspirates. Macnaughton and colleagues [Macnaughton, 1982; Macnaughton et al., 1983; Isaacs et al., 1983] have developed and evaluated and enzyme-linked immunosorbent assay based upon HCV, type specific rabbit antisera. This test is sensitive and requires only a single sample of clinical material. The development of more specific reagents, for example by recombinant DNA technology, could make this approach extremely useful as a diagnostic tool.

As an alternative to the approaches described above, we have recently developed a method for the detection of HCV229E nucleic acid in clinical material [Myint et al., 1989]. This approach is particularly suitable for viruses which are difficult to culture in vitro and it should also be relatively insensitive to serotypic variation. The method we have chosen is based upon filter hybridization using a radioactively labelled nucleic acid probe derived from HCV229E sequences located at the 3' end of the genome. In this paper we provide a molecular characterization of this probe and evaluate its sensitivity and specificity.

**MATERIALS AND METHODS**

**Viruses and Cells**

The HCV229E virus used in these studies was derived from the original isolate made by Hamre and Procknow [1966]. After isolation in secondary human embryonic kidney cells the virus was passaged in organ culture and human volunteers. In 1976, the virus was adapted to culture in MRC-5 cells and subsequently C16 cells [Phillpots, 1983]. The adapted virus was titrated by limiting dilution and the supernatant from a primary virus stock. Further stocks were obtained by propagation at low multiplicities of infection (moi) in C16 cells at 33°C. HCV229E-like viruses (HCV LP and HCV Killick) were propagated and titrated in C16 cells at 33°C. HCV043 was propagated in suckling mouse C16 cells at 33°C. HCV229E-like viruses (HCV LP and HCV Killick) were propagated and titrated in C16 cells [Phillpots, 1983]. The adapted virus was titrated at the 3' end of the genome. In this way, the plasmid pSM/F1, with an HCV-specific cDNA insert of 1.6 kb, was identified.

For the construction of a vector suitable for the synthesis of single-strand (ss) RNA probes (riboprobes), the cDNA insert of pSM/F1 was purified and ligated into the Pst1 site of the pGEM-1 vector (Promega, Heidelberg). This DNA was then used to transform E. coli HB101 cells and a recombinant clone, pSM/FG1, was identified by colony hybridization using the 32P-labeled cDNA insert of pSM/F1. The orientation of the cDNA insert in pSM/FG1 was determined by synthesizing strand-specific riboprobes (see below) by using either T7 or SP6 RNA polymerase, followed by hybridization to Northern blots of poly A RNA from HCV229E infected cells.

For the construction of a vector suitable for in vitro mRNA synthesis, the cDNA insert of pSM/F1 was purified and digested with Acc1. The 1.6 kb Pst1-Acc1 DNA fragment was then ligated at the Pst1 site to Pst1-BamH1 digested, dephosphorylated pGEM1 DNA. The linear molecule was treated with the Klenow fragment of DNA polymerase, ligated, and used to transform competent E. coli TG1 cells. A clone containing the recombinant plasmid pSM/FGM1 was identified by restriction enzyme analysis of plasmid DNA. Plasmid purifications, agarose gel electrophoresis, electroelutions, colony hybridizations, and standard recombinant DNA procedures were performed as described by Maniatis et al. [1982].

**Sequence Analysis**

Random subclones of the pSM/F1 cDNA insert were generated by sonication and subcloning into Smal cut, phosphatase treated M13 mp8 DNA. Sequencing was carried out by using the chain termination method [Sanger et al., 1977] with the M13 universal primer or HCV229E-specific oligonucleotide primers. Oligonucleotides were synthesized using phosphoramidite chemistry on a Cyclone DNA synthesizer and purified by gel electrophoresis. The sequence presented was determined completely on both cDNA strands. Sequence data were assembled by the programs of Staden [1982] and analysed by the UWGC software [Devereux et al., 1984]. The construction of the plasmid pSM/FGM1 was checked by sequencing ds plasmid DNA by using an oligonucleotide, 5' TTG AAC ATT CCA ATA GGC 3', which is complementary to a region 165–183 bases from the 5' end of the HCV229E nucleocapsid gene (Fig. 2) and modified T7 polymerase (Pharmacia, Freiburg).
**In Vitro Transcription and Translation**

The pSM/FGM1 plasmid was linearized with PstI or NarI and transcripts were synthesized by using T7 RNA polymerase [Melton et al., 1984] in the presence of the dinucleotide m7(5')Gppp(5')G to provide a cap structure [Contreras et al., 1982]. The transcription reactions were treated with DNase I (RQI DNase I, 1 U/µg DNA, 15 min, 37°C) and deproteinized by phenol extraction, and the RNA products were precipitated twice from 4M ammonium acetate with 70% ethanol at −20°C. The RNAs were dissolved in H2O. For in vitro translations 0.5–1.0 µg of in vitro synthesized RNA or poly A RNA from uninfected C16 or HCV229E infected C16 cells was translated in a reticulocyte lysate in the presence of 35S methionine as previously described [Siddell, 1983]. Translation products were analysed on linear 15% polyacrylamide-SDS gels [Laemmli, 1970]. The gels were fixed and stained, and the labelled polypeptides were detected by autoradiography. The molecular weight markers used were myosin (mol. wt. 200,000), phosphorylase b (mol. wt. 97,000), bovine serum albumin (mol. wt. 66,000), ovalbumin (mol. wt. 46,000), carbonic anhydrase (mol. wt. 30,000), and lysozyme (mol. wt. 14,300).

**Slot and Northern Blotting**

For slot blotting varying amounts of poly A RNA from HCV229E infected C16 cells were denatured at 100°C in 100 µl of H2O, mixed with an equal volume of 10 × SSC (1 × SSC is 150 mM NaCl, 10 mM sodium acetate, pH 7.0), and applied directly to nitrocellulose filters. For Northern blotting poly A RNA from HCV229E infected C16 cells was electrophoresed in 1% agarose gels containing formaldehyde, subjected to mild alkaline hydrolysis in situ, and transferred to nitrocellulose filters [Maniatis et al., 1982]. After baking at 80°C for 2 hours the filters were prehybridized for 4 hours at 42°C in either 50% formamide, 1 × Denhart’s solution, 1% SDS, 5 × SSC, and 250 µg/ml of denatured herring sperm DNA (DNA probes) or 50% formamide, 50 mM sodium pyrophosphate pH 6.5, 5 × SSC, 0.1% SDS, 0.05% Ficoll, 0.05% PVP, and 200 µg/ml of denatured herring sperm DNA (riboprobes). Hybridizations were then performed under the same conditions for 16 hours. The filters were washed twice for 30 min in 0.2 × SSC/0.1% SDS at 55°C (DNA probes) or 0.1 × SSC/0.1% SDS at 65°C (riboprobes) and autoradiographed.

**Hybridization to Respiratory Viruses**

32P labelled DNA probes were made by nick transcription [Rigby et al., 1977] or by using the Multiprime system (Amersham, High Wycombe) according to the manufacturer’s instructions. Specific activities of approximately 106 and 5 × 108 dpm/µg DNA respectively, were obtained. Strand-specific RNA probes were generated by using a reaction containing 4 µl of 5 × transcription buffer (0.2 M Tris HCl pH 7.5, 30 mM MgCl2, 50 mM NaCl, 10 mM spermidine) 2 µl 100 mM DTT, 0.8 µl RNAsin (25 U/µl), 1 µl 2.5 mM ATP, 1 µl 2.5 mM GTP, 1 µl 2.5 mM UTP, 2.2 µl 100 µM CTP, 2 µl (1 µg) HindIII-linearized pSM/FG1 DNA, 5 µl 32P CTP (10 µCi/µL), and 1 µl of T7 polymerase (10 U/µl). The reaction was incubated at 37°C for 1 hour before 1 µl of RQI DNase (1 U/µl) was added. After a further 15 min the reaction was deproteinized by phenol extraction. The aqueous phase was precipitated at −20°C by the addition of ammonium acetate and ethanol. After centrifugation the RNA precipitate was resuspended in 10 mM Tris HCl pH 7.5, 1 mM EDTA. Specific activities of approximately 5 × 106 dpm/µg RNA were routinely obtained. 32P labelled probes were denatured at 100°C for 2 min prior to inclusion in the hybridization buffer.

**RESULTS**

**Sequence Analysis of the pSM/F1 cDNA Insert**

The plasmid pSM/F1 was identified as an HCV-specific clone by hybridization to poly A RNA from HCV229E infected C16 cells. Figure 1 shows that the cDNA insert of pSM/F1 hybridizes to seven intracellular RNAs which have been previously identified as HCV229E virus specific by metabolic labelling in the presence of actinomycin D [Schreiber et al., 1989], oligonucleotide hybridization, and sequence analysis [Raabe et al., 1990]. This locates the pSM/F1 insert sequences at the 3′ end of the HCV229E genome.

The nucleotide sequence of 1,576 bases from the pSM/F1 cDNA insert is shown in Figure 2. Recently, Schreiber et al. [1989] have reported a sequence for the HCV229E nucleocapsid gene and leader RNA and by comparison to their data it is evident that the pSM/F1 cDNA insert represents a copy of the HCV229E mRNA 7. The main open reading frame (ORF) of this cDNA copy (positions 66 to 1235 in Fig. 2) potentially encodes a polypeptide of 389 amino acids (mol. wt. 43,450). Compared to the sequence reported by Schreiber et al. [1989], there are 13 nucleotide differences in the coding region (resulting in nine amino acid changes) and an additional stretch of 42 bases in the 3′ non-coding region of the pSM/F1 cDNA.

**In Vitro Transcription and Translation of the HCV229E Nucleocapsid Gene**

In order to confirm the identity of the pSM/F1 insert we have performed in vitro transcription and translation experiments. Figure 3 shows the in vitro transla-
Fig. 1. Northern blot analysis of HCV229E intracellular RNAs. Poly A RNA from HCV229E infected C16 cells (A) or uninfected C16 cells (B) was probed with the cDNA insert of pSM/F1 labelled with 32P by nick translation. Autoradiography was for 16 hours. The HCV-specific intracellular RNAs are numbered according to Raabe et al. [1990].

Fig. 2. Nucleotide sequence of the HCV229E nucleocapsid protein gene. The nucleotide sequence and the derived amino acid sequence of the pSM/F1 copy of the HCV229E N gene are shown. Differences from the HCV229E N gene sequence recently reported by Schreiber et al. [1989] are indicated above the pSM/F1 sequence. Deleted nucleotides are indicated by an asterisk.

Sensitivity and Specificity of the pSM/FG1 Probe

In order to estimate the amounts of HCV229E RNA which can be conveniently detected by using the pSM/FG1 probe we have performed slot blot hybridizations to varying amounts of poly A RNA from HCV229E infected C16 cells. Figure 4 shows that using either an pSM/FG1 derived Multiprime probe or a riboprobe, a positive hybridization signal can be obtained with as little as 1 ng of poly A RNA under the conditions used. The absolute amount of HCV229E-specific RNA in the poly A RNA fraction is not known, but on the basis of ethidium bromide stained gels we estimate that it does not exceed 5%. This would mean that approximately 50 pg of HCV229E RNA can be detected. The pSM/FG1 derived riboprobe appeared to be marginally more sensitive than the Multiprime probe.

A second major consideration is the specificity of the pSM/FG1 probe. To investigate this aspect we performed slot blot hybridizations with a pSM/FG1 derived riboprobe and a variety of viruses which are associated with respiratory illness. These hybridizations were performed under conditions which simulated the
Fig. 3. In vitro translation of natural and synthetic HCV229E mRNAs encoding the nucleocapsid protein. Poly A RNA or RNA transcribed in vitro was translated in a reticulocyte lysate and the products were analysed on an SDS polyacrylamide gel.

Lane:
A: Mol. wt. markers.
B: H₂O.
C: 0.5 μg poly A RNA from uninfected C16 cells.
D: 0.5 μg poly A RNA from HCV229E infected C16 cells.
E: 0.5 μg of RNA transcribed in vitro from the pSM/FGM1 plasmid in the presence of m7(5')Gppp(5')G.
F: 1.0 μg of RNA transcribed in vitro from the plasmid pSM/FGM1 in the absence of m7(5')Gppp(5')G.
G: 0.5 μg of RNA transcribed in vitro from the pSM/FGM1 plasmid in the absence of m7(5')Gppp(5')G.
H: 1.0 μg of RNA transcribed in vitro from the pSM/FGM1 plasmid in the absence of m7(5')Gppp(5')G. Autoradiography was for 8 hours.

Finally, we wished to ascertain the sensitivity of the pSM/FG1 hybridization probe under conditions that would approximate those in a diagnostic situation. To do this nasal washings were inoculated with tissue culture supernatant containing different amounts of infectious HCV229E virus. The result is shown in Figure 6 and indicates that nasal washings with virus titres as diagnosis of infection in clinical material; i.e., the viruses were added to nasal washing. The viruses which were tested are listed in Table I, together with the titres of infectious virus in the material used to inoculate the nasal washings. As can be seen, the titres used greatly exceed those which would be expected in nasal washings from patients. The result of this experiment, shown in Figure 5, confirms that the pSM/FG1 probe is specific for HCV229E-like viruses.

Table I. Titres of Respiratory Viruses

| Virus     | Titre (TCID₅₀/ml) | Virus     | Titre (TCID₅₀/ml) |
|-----------|------------------|-----------|------------------|
| HRV 1A    | 10⁶              | HRV 49    | 10⁴              |
| HRV 1B    | 10⁵              | HRV 61    | > 10⁶            |
| HRV 2     | > 10⁶            | HRV 63    | > 10⁸            |
| HRV 3     | 10⁷              | HRV 68    | > 10⁸            |
| HRV 4     | 10⁷              | HRV 71    | > 10⁸            |
| HRV 5     | 10⁷              | HRV 72    | > 10⁸            |
| HRV 6     | 10⁵              | HRV 75    | > 10⁸            |
| HRV 9     | 10⁷              | HRV 83    | > 10⁸            |
| HRV 13    | 10⁷              | HRV 88    | > 10⁸            |
| HRV 14    | 10⁸              | HCVOC43   | 32³              |
| HRV 16    | 10⁷              | HCV LP    |                  |
| HRV 23    | > 10⁸            | HCV229E   | 10⁷              |
| HRV 29    | 10⁷              | HCKVillick| 10⁸              |
| HRV 30    | 10⁶              | PF2       |                  |
| HRV 31    | 10⁷              | PF3       | Not available    |
| HRV 32    | 10⁶              | PF4A      | 10⁸              |
| HRV 34    | > 10⁸            | PF4B      | Not available    |
| HRV 43    | 10⁶              | INF.A/E/40/83 | 10⁶b            |
| HRV 45    | 10⁶              | INF.A/C/10/78 | 10⁶b            |
| HRV 47    | 10⁶              | INF.B/B/11/78 | 10⁶b            |
| HRV 48    | 10⁶              | INF.B/B/222/79 | 10⁶b            |

*HRV, human rhinovirus; HCV, human coronavirus; PF, human parainfluenzavirus; INF, human influenzavirus.
³Haemagglutination titre, expressed as reciprocal of end point dilution.
⁶Plaque forming units per ml.
Fig. 5. Slot blot hybridization of a pSM/FG1 derived riboprobe with respiratory viruses. Nasal washings inoculated with the viruses listed in Table I were probed in triplicate (1, 2, 3) as described in the Materials and Methods. Autoradiography was for 6 hours at room temperature. HRV, human rhinovirus; HCV, human coronavirus; PF, parainfluenzavirus; INF, influenzavirus.

low as $10^1$ to $10^2$ TCID$_{50}$/ml would give a positive hybridization signal. This result is in agreement with earlier experiments involving clinical material from experimentally infected volunteers [Myint et al., 1989].

DISCUSSION

This study provides a detailed characterization of a nucleic acid hybridization probe which we have developed for the diagnosis of human coronavirus HCV229E infections. The most important aspects which have to be considered are the specificity of the probe, its sensitivity, and its potential application as a routine diagnostic tool.

The specificity of the pSM/FG1 probe can be judged in two ways. Firstly, our Northern blot analysis, nucleotide sequence, and in vitro transcription/translation experiments have shown that the pSM/F1 cDNA insert represents a copy of the HCV229E nucleocapsid gene. It is therefore possible to use this sequence data to predict any potential cross hybridization with other viral sequences. As an example, a comparison of the

Fig. 6. Slot blot hybridization of a pSM/FG1 derived riboprobe with HCV229E virus. Nasal washings inoculated with different amounts of infectious HCV229E virus were probed in duplicate (1, 2) as described in Materials and Methods. Autoradiography was for 24 hours using Cronex 2 film and two intensifying screens at $-70^\circ$C.
One aspect which we have not addressed in this study is the effect that intraspecific genetic variability might have on the diagnosis of HCV229E infection by hybridization probes. The sequence data presented here and by Schreiber et al. [1989] show that, as expected, point mutations and insertion/deletion occur in the HCV229E N gene. Also, it is known that at least for one coronavirus, the murine hepatitis virus (MHV), recombination occurs in vivo and in vitro at relatively high frequencies [Makino et al., 1986a,b; Keck et al., 1988]. Thus it would seem prudent to exercise caution, especially if, for example, short oligonucleotides were used as hybridisation probes. However, we believe that with "gene" probes such as pSM/FG1, there is always likely to be sufficient sequence homology to provide for hybridization.

The final consideration is the application of nucleic acid hybridization as a routine procedure for the diagnosis of HCV229E infection. A major advantage of this approach is its speed. The test can be conducted within 24 hours and with the advent of antiviral chemotherapy a rapid diagnosis would be imperative prior to specific medication. A second advantage is that the approach is independent of whether or not virus is bound by antibody or drugs. This could be important, for example, when virus infection persists in the presence of antibody or in situations where virus infection has to be monitored following medication. At the present time, the major disadvantage of the method is the radioactive nature of the probe. However, a number of non-radioactive detection systems are currently being developed and this modification would make the hybridisation approach suitable for general diagnostic use.

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