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 × 10³) [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-

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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 well with one focus of infection was taken as the 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. HCVO43 was propagated in suckling mouse brain and the haemagglutinin titre determined as described by Hierholzer et al. [1972].

Human rhinoviruses were grown and titrated in Ohio HeLa cells and influenza A and B virus in Madin-Darby canine kidney (MDCK) cells, as described by Al-Nakib et al. [1986]. Human parainfluenza viruses (2, 3, 4A, and 4B) were propagated and titrated in BS-C-1 cells and haemagglutinin titres were determined as described by Guskey and Bergstrom [1981]. Infectious virus titrations were performed by end point dilution and calculated as described by Reed and Muench [1938].

cDNA Synthesis and Cloning

Cytoplasmic RNA was isolated by standard procedures from C16 cells which had been infected 48 hours previously with HCV229E at an moi of 3. Polyadenylated RNA (poly A RNA) was selected by chromatography on poly U Sepharose and cDNA was synthesized essentially as described by Gubler and Hoffman [1983], using oligo dT as first-strand primer. The double strand (ds) cDNA was cloned into the Pst1 site of the plasmid pAT153 by homopolymeric tailing and used to transform competent *E. coli* BMH 71-18 cells. A library of transformants containing plasmids with cDNA inserts of 1–2 kb was selected and individual cDNA inserts were labelled with ^{32}P by nick translation. The cDNA inserts were then hybridized to Northern blots of poly A RNA from HCV229E infected or uninfected C16 cells. In this way, the plasmid pSM/F1, with an HCV-specific cDNA insert of 1.65 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 ^{32}P-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 Sma1 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 UWGCG 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 GCC 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 Pst1 or Nar1 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, 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 H_2O. 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. 69,000), 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 H_2O, 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 translation [Rigby et al., 1977] or by using the Multiprime system (Amersham, High Wycombe) according to the manufacturer's instructions. Specific activities of approximately 10^7 and 5×10^8 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 $MgCl_2$, 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 5×10^8 dpm/μg RNA were routinely obtained. [32P] labelled probes were denatured at 100°C for 2 min prior to inclusion in the hybridization buffer.

Tissue culture supernatants (200 μl) containing defined titres of a variety of respiratory viruses were mixed with nasal washings (300 μl) obtained from healthy volunteers. The inoculated nasal washings were then mixed with 50 μl of 10 × proteinase K buffer (10 mM Tris HCl pH 7.8, 0.5 mM EDTA, 0.5% SDS) and 10 μl proteinase K (10 mg/ml) and incubated at 37°C for 20 min. After addition of 500 μl 6.15M formaldehyde/10 × SSC, aliquots of 350 μl were applied to nitrocellulose filters which were then baked at 80°C for 2 hours. The conditions for prehybridization and hybridization were as described above for slot blotting with riboprobes.

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-
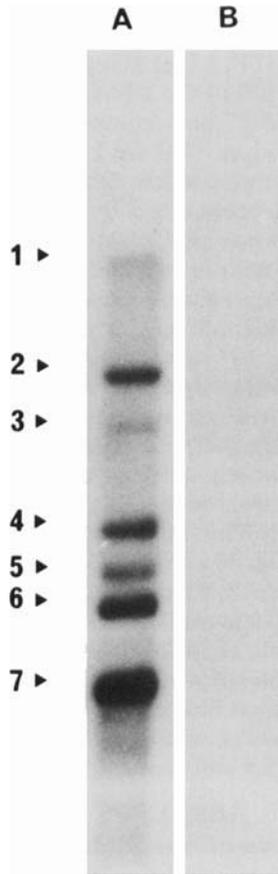
Fig. 1. Northern blot analysis of HCV229E intracellular RNAs. Poly A RNA from HCV229E infected C16 cells (A) or uninfected C16 cells (B) was probed with the cDNA insert of pSM/F1 labelled with ^{32}P by nick translation. Autoradiography was for 16 hours. The HCV-specific intracellular RNAs are numbered 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.

tion products of poly A RNA from uninfected C15 cells (lane C) and HCV229E infected C16 cells (lane D). The two major infection-specific polypeptides which can be identified have apparent molecular weights of 50,000 and 23,000, representing the N and M proteins, respectively [Schmidt and Kenny, 1982]. The in vitro translation of capped, synthetic RNA derived from pSM/FGM1 (lanes E and F) also results in a major product of 50,000 mol. wt. These data provide strong evidence for the identity of the pSM/F1 insert as a cDNA copy of the HCV229E mRNA7. However, it is also clear in Figure 3 that the translation of both natural and synthetic mRNAs results in a second major product of 44,000 mol. wt., the molecular weight of the N gene product predicted from the amino acid sequence alone. The relationship of the 44,000 and 50,000 mol. wt. species is not known at the moment, but it seems likely that they represent related polypeptides, at least one of which has been post-translationally modified. It is striking that the in vitro translation of a non-capped, synthetic RNA derived from pSM/FGM1 (lanes G and H) resulted in only the 50,000 mol. wt. product. Experiments are in progress to resolve these questions.

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 pSMFG1 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
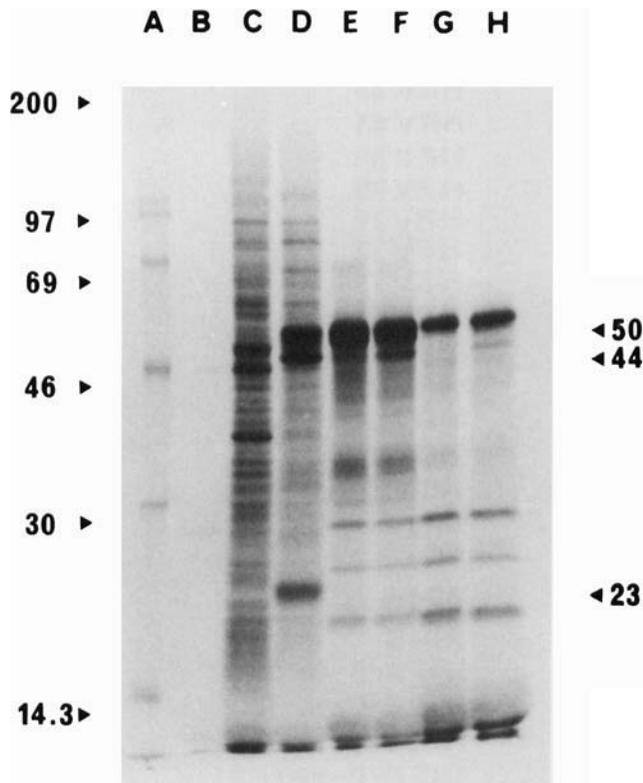
Fig. 3. In vitro translation of natural and synthetic HCV229E mRNAs encoding the nucleocapsid protein. Poly A RNA or RNA transcribed in vitro was translated in a reticulocyte lysate and the products were analysed on an SDS polyacrylamide gel.
Lane:
A: Mol. wt. markers.
B: H_2O.
C: 0.5 μg poly A RNA from 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 presence 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 48 hours.

Fig. 4. Slot blot hybridization of pSM/FG1 derived probes with poly A RNA from HCV229E infected C16 cells. Varying amounts of poly A RNA were probed as described in Materials and Methods by using either a ds DNA Multiprime probe (A) or an ss RNA riboprobe (B). Autoradiography was for 8 hours using Cronex 2 film and one intensifying screen at −70°C.

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.

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

TABLE I. Titres of Respiratory Viruses*

Virus	Titre (TCID$_{50}$/ml)	Virus	Titre (TCID$_{50}$/ml)
HRV 1A	10^6	HRV 49	10^5
HRV 1B	10^5	HRV 61	$> 10^8$
HRV 2	$> 10^8$	HRV 63	$> 10^8$
HRV 3	10^7	HRV 68	$> 10^8$
HRV 4	10^7	HRV 71	$> 10^8$
HRV 5	10^6	HRV 72	$> 10^8$
HRV 6	10^5	HRV 75	$> 10^8$
HRV 9	10^8	HRV 83	$> 10^8$
HRV 13	10^7	HRV 88	$> 10^8$
HRV 14	10^8	HCVOC43	32^a
HRV 16	10^7	HCV LP	10^7
HRV 23	$> 10^8$	HCV229E	10^7
HRV 29	10^7	HCVKillick	10^5
HRV 30	10^6	PF2	512^a
HRV 31	10^7	PF3	Not available
HRV 32	10^6	PF4A	10^4
HRV 34	$> 10^8$	PF4B	Not available
HRV 43	10^6	INF.A/E/40/83	10^{8b}
HRV 45	10^6	INF.A/C/10/78	10^{8b}
HRV 47	10^5	INF.B/B/11/78	10^{8b}
HRV 48	10^6	INF.B/B/222/79	10^{9b}

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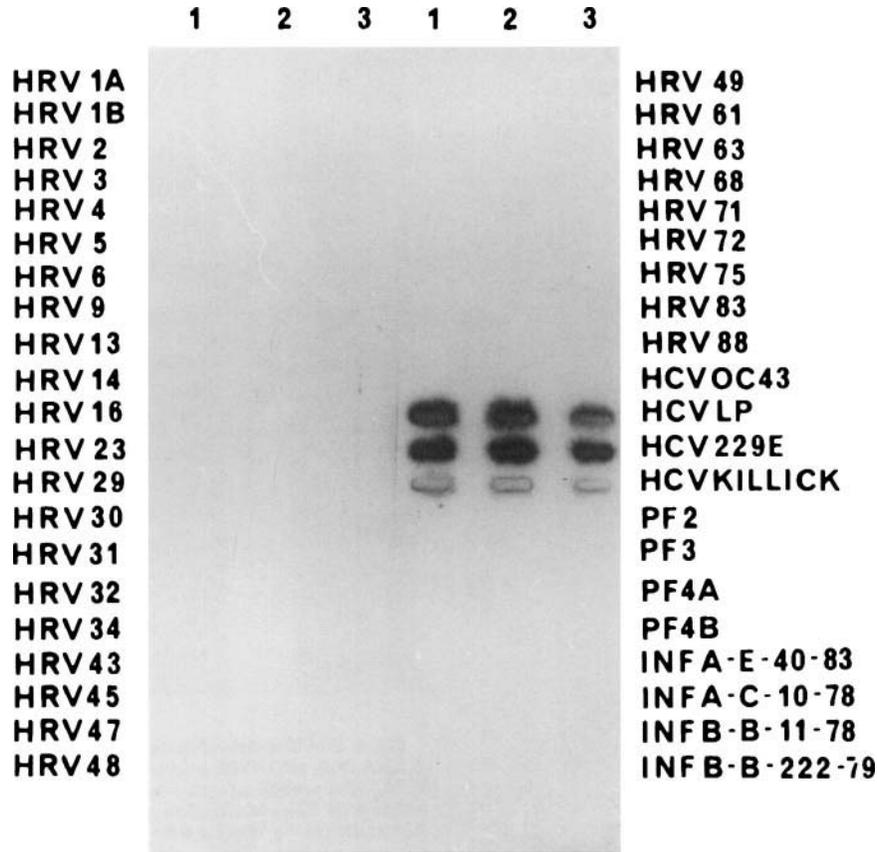
Fig. 5. Slot blot hybridization of a pSM/FG1 derived riboprobe with respiratory viruses. Nasal washings inoculated with the viruses listed in Table I were probed in triplicate (1, 2, 3) as described in the Materials and Methods. Autoradiography was for 6 hours at room temperature. HRV, human rhinovirus;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

A

229E nucleotide

B

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K  L  H (F)(V)(V)(L)(G)(T)(G)(P)(H)  K  D  A  K  F  R  E  R  V  E (G)(V)  v (W)(V)(A) V
AAGCTGCATTTTTATTTATCTTGGCACAGGACCCCATAAAGATGCAAAATTTAGAGAGCGTGTTGAAGGTGTCGTCTGGGTTGCTGTT
* ******  *****  **  *******  ***     *  *  *  *   *  ****  **  ***  ******  ***  *
CGATGGTATTTTTACTTATCTCGGAAGAACGACCTGCCATGCTAAAGACCAGTACGGCACCGATATTGACGGAGTCTACTCGGTCGGTAGT
R  W  Y (F)(S)(Y)(L)(G)(G)(G)(S)(P)(H)  A  K  D  Q  Y  G  T  D  I  D (G)(V)  Y (W)(V)(A) S
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Fig. 7. A comparison of the HCV229E and HCVOC43 N gene sequences. The coding sequences of the HCV229E and HCVOC43 N genes [this paper; Kamahora et al., 1989] were compared by using the UWGCG program COMPARE and the result was displayed by using the program DOTPLOT (**A**). The sequence comparison of one region (229E nucleotides 229 to 315 and OC43 nucleotides 364 to 450) which displayed significant similarity is shown in detail (**B**). Identical nucleotides (*) and amino acids (circled) are indicated.

HCV229E N protein gene with the HCVOC43 N protein gene [Kamahora et al., 1989] is shown in Figure 7. This comparison predicts that the potential for cross hybridization is very low. In a region corresponding to the nucleotides 229 to 315 (229E) and 364 to 450 (OC43) of the N gene, the nucleotide sequence similarity is significant, but even so would not lead to cross hybridization under the conditions used in our experiments. Secondly, the specificity can be determined empirically. In the experiments reported here and in other unpublished studies we have observed hybridization of the pSM/FG1 probe only with nucleic acids derived from viruses defined serologically as HCV229E-like.

The second important aspect is the sensitivity of the pSMFG1 probe. Our data indicate that approximately 50 pg of intracellular viral RNA can be detected under the conditions used. Taking into account the size of the HCV genome and the probe, this would translate into approximately 10^4 genome equivalents. It was therefore quite surprising to find that using essentially similar conditions, a clear hybridization signal could be obtained from inoculated nasal washings containing only 10^1 $TCID_{50}$ of virus. The most probable explanation for this discrepancy is that in tissue-culture-propagated virus the ratio of infectious to non-infectious viral RNA is low. Our earlier experiments have shown that in the course of a natural infection there is no correlation between the infectious virus titre in nasal washings and the amount of viral RNA as determined by hybridization [Myint et al., 1989].

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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