Detection of Human Rhinoviruses and Their Molecular Relationship Using cDNA Probes

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We describe here a cDNA:RNA hybridization system for the study of human rhinoviruses. We have constructed an M13 probe from the 5' end of the genome of rhinovirus 14 (HRV-14) and used this to detect directly viral RNA. Of the 56 human rhinoviruses so far investigated 54 or 96.4% gave clearly positive hybridization signals. However, the strength of this signal depended very much on the molecular relationship of these viruses. Thus, HRV-3, 4, 17, 72, and, to a slightly lesser extent, HRV-2, 6, 9, 13, 19, 31, 42, 49, 64, and 69 appear to be closely related to HRV-14 whereas HRV-5, 7, 8, 16, 32, 40, 45, 55, 56, 63, 80, 82, and 85 appear to be relatively divergent. Further, evidence is provided in this study that indicates that it would be feasible to use cDNA probes to detect human rhinoviruses in nasal washings. However, the sensitivity of detection was clearly affected by both the inclusion of inhibitors of endogenous RNase activity in the RNA extraction mixture and also in the method of extracting the viral RNA. From reconstruction experiments in nasal washings and under optimal conditions, we can detect virus at $10^{2.8}$ TCID₅₀/ml.

Key words: rhinoviruses, cDNA probe, hybridization

INTRODUCTION

Human rhinoviruses are the major causative agents of the common cold. Recent surveys in the United States suggest that rhinovirus common colds are responsible for one-third to one-half of all acute respiratory illnesses and it is estimated that these occur at rates of two to five per person per year [for review see Couch, 1984]. These viruses are thus a major cause of morbidity and economic loss especially in the developed world. With the advent of antiviral chemotherapy a rapid virus diagnosis would be imperative prior to specific medication. However, as yet, human rhinovi-

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ruses can only be diagnosed by isolation in tissue cultures. Virus replication in a sensitive cell line may take up to 8 days before a cytopathic effect appears. Further, the sensitivity of cell cultures to the various serotypes is quite variable. With the great multiplicity of serotypes (over 115) detection of these viruses by serological means is, at present, impracticable.

We have, therefore, developed an alternative approach to rhinovirus detection: that of viral RNA detection using cDNA:RNA hybridization procedures. We have recently cloned and sequenced the entire genome of human rhinovirus 14 (HRV-14) [Stanway et al, 1984]. The first 624 nucleotides from the 5' end region of the genome encode no open-reading frame of significant length and in common with other picornaviruses are presumed to have a noncoding function. Somewhat surprisingly, we found that this region was highly homologous to the corresponding region of the enterovirus, poliovirus, at the nucleotide sequence level. For this reason we think that it is likely to be relatively highly conserved within the rhinovirus genus and we would expect a cDNA probe from this region of the HRV-14 genome to cross-hybridize with RNA from other rhinoviruses. We, therefore, constructed a single-stranded M13 template containing the first 800 nucleotides from the 5' end terminus, comprising the whole of the noncoding region plus some of the VP4 coding sequence. We used this template to generate a cDNA probe that was used to establish a cDNA:RNA hybridization system for rhinovirus detection. In a preliminary study we found that this probe does indeed cross-hybridize with a number of human rhinoviruses indicating the feasibility of cDNA:RNA hybridization for rhinovirus detection [Al-Nakib et al, 1986]. We have now extended these studies to include a larger series of human rhinoviruses (totalling some 56 viruses) and looked in more detail at the molecular relationship between these viruses, the limits of detection, and the feasibility of applying these procedures for the direct detection of viral RNA in nasal washings. We have also investigated different procedures for extracting rhinovirus RNA and how these may affect the sensitivity of detection in nasal washings.

MATERIALS AND METHODS

Propagation of Viruses

Stock rhinoviruses and enteroviruses (echo and coxsackie) were prepared by propagation in Ohio HeLa cells as described earlier [Tyrrell, 1965]. Viruses were harvested when the cytopathic effect was approximately 80% of the cell layer. Virus was released from the cells by repeated cycles of freeze-thawing (a total of three cycles). Each stock was titrated in Ohio HeLa cells using log dilutions. Titrations were carried out in microtitre plates (Nunc/Delta). Influenza A (A/Eng/40/83) and B viruses were propagated and titrated in Madin-Darby canine kidney (MDCK) cells and similarly titrated in microtitre plates, while coronavirus strain 229E was prepared and titrated in modified MRC-C cells as described previously [Phillpotts, 1983].

Extraction of Viral Nucleic Acid

Viral nucleic acid was extracted by first treating each specimen with sodium dodecyl sulphate (SDS) to a final concentration of 0.5%. Samples were then treated with proteinase K (BRL) (1.6 mg/ml) and the mixture incubated at 37°C for 20 min. It was extracted twice with a mixture containing phenol:chloroform:isoamyl alcohol:8-hydroxyquinoline in a ratio of 100:100:4:0.1. The aqueous phase was collected

each time and the RNA precipitated from the aqueous phase by adding sodium acetate (pH 6.0) and to a final concentration of 200 mM and 2.5 volumes of ethyl alcohol. The precipitate was allowed to form by incubating the mixture at -20°C overnight and then pelleted by centrifugation at 13,000 rpm for 10 min using Eppendorf tubes. The pellet was then dissolved in 25–50 μl of Tris-EDTA buffer (10 mM Tris HCl, pH 7.4, 1 mM EDTA).

Two further procedures were investigated for RNA extraction following virus reconstitution in nasal washings. The first involves the addition to the sample of an equal volume of a 3:2 mixture of $20 \times \text{SSC}$ —37% formaldehyde while in the second method an equal volume of $20 \times \text{SSC}$ was added. Both these procedures have been described earlier for other picornavirus RNA fixation prior to hybridization [Rotbart et al, 1984, 1985].

In all preparations we have included at the beginning of each extraction vanadyl-ribonucleoside complexes (VRC) at a concentration of 20 mM as inhibitors of endogenous RNase activity. We have found this to be essential for improving the sensitivity of RNA detection.

Immobilization of Viral RNA Onto Nitrocellulose Filters

Using a manifold system (Schleicher and Schuell) the extracted viral RNA (10 μl per well in duplicate) was immobilized onto nitrocellulose filters (Schleicher and Schuell; BA-85) that had been washed three times in distilled water and then soaked with $20 \times$ sodium chloride/sodium citrate (SSC) buffer. The filters were allowed to dry for a few minutes and then removed from the manifold and baked for 4 hr at 80°C using a vacuum oven. Filters were either processed immediately or stored at room temperature in an air tight box.

Construction and Labelling of the Probe

Details of the procedure for constructing and labelling our M13 probe will be described elsewhere [Al-Nakib et al, 1986]. Briefly, a single-stranded M13 template with HRV-14 cDNA from the 5' end of the genome inserted into the PstI and BamHI sites of the multiple cloning region was used to generate a second cDNA strand labelled with high specific activity ^{32}P -dATP ($\sim 3000 \mu\text{Ci}/\text{mM}$, Amersham International).

Prehybridization

The prehybridization buffer consisted of 50% v/v formamide (Sigma), $5 \times \text{SSC}$, 50 mM sodium phosphate (pH 6.5), 0.25 mg/ml of sonicated denatured salmon sperm DNA (Sigma), 0.02% bovine serum albumin, 0.02% Ficoll (Sigma, type 400), 0.02% polyvinylpyrrolidone (Sigma, PVP-360). Filters were treated with this prehybridization buffer (making sure that all air bubbles were totally excluded) for a period of 8–20 hr at 42°C .

Hybridization

The prehybridization buffer was removed and filters were treated this time with the hybridization buffer (consisting of four parts of the prehybridization buffer and one part of 50% w/v dextran sulphate, to give a final concentration of 40% formamide and 10% dextran sulphate) and denatured ^{32}P -labelled probe (1×10^7 to 1×10^8 cpm/ μg of DNA). The filters were incubated for approximately 16 hr at 42°C and

then washed twice for 10 min in $2 \times \text{SSC}$ at room temperature. This was followed by a further two washes, this time for 40 min each, in $1 \times \text{SSC}$ at 42°C . Filters were air-dried and exposed to an X-ray film (Fujifilm RX) at -70°C for approximately 48 hr using an X-ray cassette with intensifying screens. Probes prepared using lower specific activity dATP (200–300 μCi) often required a longer exposure period to the X-ray film, ranging from 4–14 days.

RESULTS

The Detection of Various Rhinovirus Serotypes Using cDNA Probes

The results of our experiments on the hybridization of the HRV-14 5' end probe to the panel of rhinoviruses are shown in Figure 1. Using this probe 54 of 56 (96.4%) of the human rhinoviruses investigated gave clearly positive hybridization signals.

Fig. 1. Detection of human rhinoviruses by cDNA:RNA hybridization.

Two other human rhinoviruses, HRV-45 and HRV-48, gave weak hybridization signals. It is noteworthy that our probe also cross-hybridized with two animal rhinoviruses, calf rhinovirus SD1 and bovine rhinovirus EC11. Furthermore, it detected coxsackie A21 virus but not echovirus type 1. None of the control viruses, such as influenza A, B, coronavirus 229E, or parainfluenza type 3, reoviruses 1 and 3 (data not shown) gave any positive hybridization signals. These data were reproduced a number of times and were shown to be very consistent.

The Molecular Relationship Between Human Rhinoviruses

It is probable that the strength of the hybridization signal will reflect the titre of the virus. However, as can be seen from Table I, which summarises the extent of hybridization and the virus titres, this is not the only factor. For example, HRV-17, with a titre of $10^{5.5}$ TCID₅₀/ml gave a very strong signal (++++), while HRV-41 with a titre of 10^7 TCID₅₀/ml gave a signal of about half the strength. Similarly, HRV-72 with a titre of $10^{3.5}$ TCID₅₀/ml gave a much stronger signal than HRV-32, which had a titre of $10^{6.5}$ TCID₅₀/ml. These data suggest that although there is considerable homology among the different rhinovirus serotypes, the degree of this homology varies from serotype to serotype. Using the data summarized in Table I it is possible to arrive at a preliminary estimate of the molecular relationship among the different human rhinoviruses. Thus, it can be seen that HRV-3, 4, 17, and 72 are very closely related to HRV-14, at least in relation to the 5' noncoding region of the genome. Also related but to a slightly lesser extent are HRV-2, 6, 9, 13, 19, 31, 42, 49, 64, and 69. Others such as HRV-5, 7, 8, 16, 32, 40, 45, 55, 56, 63, 80, 82, and 85 gave much weaker hybridization signals despite most of them having titres in excess of $10^{5.5}$ TCID₅₀/ml and these appear to be more divergent from HRV-14.

Detection of Human Rhinoviruses in Nasal Washings

Table II shows the results of virus reconstruction experiments to determine the limit of sensitivity of virus detection in nasal washings using three different procedures for extracting viral RNA. Method 1 was that of phenol/chloroform extraction, as described in this paper, while method 2 was that of direct fixation with $20 \times$ SSC—37% formaldehyde and method 3 fixation with $20 \times$ SSC only. As can be seen, RNA fixation with $20 \times$ SSC—37% formaldehyde was clearly the best procedure, detecting HRV-14 at $10^{2.8}$ TCID₅₀/ml, while that of phenol/chloroform extraction detected virus at $10^{3.5}$ TCID₅₀/ml. Treatment of samples with $20 \times$ SSC provided the least satisfactory procedure since a virus titre of $10^{4.3}$ TCID₅₀/ml was required for detection. However, in addition to detecting virus at lower titres, the strength of the hybridization signals obtained with $20 \times$ SSC—37% formaldehyde were particularly strong at all virus dilutions in nasal washings compared with phenol/chloroform extraction, indicating that more viral RNA has been immobilised onto the nitrocellulose filters. This particular feature of this procedure will undoubtedly prove important in the detection of viral RNA in clinical specimens.

DISCUSSION

In this paper we have shown that it would be feasible to detect rhinoviruses directly using cDNA:RNA hybridization procedures. This provides a new approach to the detection and study of rhinoviruses with the potential of overcoming many of

TABLE I. Detection of Rhinovirus by cDNA-RNA Hybridization Using HRV-14 ³²P-labelled Probe

Virus	TCID ₅₀ /ml	Hybridization signal ^a	Virus	TCID ₅₀ /ml	Hybridization signal ^a
HRV-1a	6.5	++	47	2.5	++
HRV-1b	5.0	++	48	4.0	(±)
HRV-2	6.5	+++	49	7.0	+++
3	7.5	++++	51	3.5	+
4	7.5	++++	55	5.5	+
5	5.0	+	56	6.5	+
6	7.5	+++	62	4.0	++
7	6.0	+	63	5.0	+
8	6.0	+	64	7.0	+++
9	7.0	+++	65	6.0	++
10	7.0	++	69	5.5	+++
11	7.5	++	70	4.5	+
12	5.5	++	72	3.5	++++
13	7.0	+++	73	2.0	++
14	7.5	++++	75	4.0	+
15	7.0	++	77	4.0	++
16	6.0	+	80	6.5	+
17	5.5	++++	81	2.5	+
18	7.0	++	82	6.0	+
19	N.D.	+++	85	6.5	+
20	6.0	++	EL	7.0	++
23	4.0	++	Calf RV SD1	1.5	+
24	5.5	++	Bovine RV EC11	1.0	++
25	4.0	++	Coxsackie A21	7.5	++
27	6.5	++	Echo 1	3.5	—
28	3.5	+	Influenza A	8.0	—
29	7.5	++	Influenza B	7.0	—
30	7.0	++	Coronavirus 229E	6.0	—
31	5.5	+++			
32	6.5	+	Uninfected	—	—
40	6.0	+	tissue		
41	7.0	++	culture		
42	6.0	+++	fluids		
44	3.5	++			
45	5.5	(±)			

^aHybridization signal: + + + +, very strong hybridization signals; + + +, strong hybridization signals; + +, good hybridization signals; +, positive hybridization signals; (±), weak hybridization signals; —, negative hybridization signals.

the short-comings of detection by propagation of virus in tissue-culture systems. An interesting finding is the fact that it would be feasible to detect a large majority of human rhinoviruses by using only one probe. However, this study also demonstrated that the sensitivity of detection using only one probe will depend very much on the degree of homology between the rhinoviruses in relation to the region of the genome from which the probe was constructed. In order to overcome this particular problem and to increase the strength of the hybridization signals, and consequently the sensitivity of detection, a mixture of probes could be used from different regions of the genome and/or from different rhinoviruses. We are, therefore, now constructing

TABLE II. Comparison of Three Procedures for Extracting Viral RNA in Nasal Washings

Method	Virus titre (TCID ₅₀ /ml) ^{a,b}					
	10 ^{4.8}	10 ^{4.3}	10 ^{3.8}	10 ^{3.3}	10 ^{2.8}	10 ^{2.3}
Phenol/chloroform	++	++	+	+	—	—
20 × SSC—37% formaldehyde	++++	+++	++	++	+	—
20 × SC	++++	+	—	—	—	—

^aVirus titre: + + + +, very strong hybridization signals; + + +, strong hybridization signals; + +, good hybridization signals; +, positive hybridization signals; —, negative hybridization signals.

^bOriginal stock virus titre was 10⁷ TCID₅₀/ml.

probes from the 3' end of the HRV-14 genome as well as probes from other human rhinoviruses to be used in the present hybridization system.

In this study we have had the opportunity to look for the first time at the molecular relationship between the different human rhinovirus serotypes, at least in the 5' end region represented by the probe. Clearly there is a considerable genomic homology between the different rhinoviruses, although the degree of homology varied. For example, in this study it was evident that HRV-3, 4, 17, and 72 and, to a slightly lesser extent, HRV-2, 6, 9, 13, 19, 31, 42, 49, 64, and 69 were closely related to HRV-14 whereas HRV-5, 7, 8, 16, 32, 40, 45, 55, 56, 80, 82, and 85 appear to be more divergent. It will be possible to construct a more detailed picture of the molecular relationships between the different rhinoviruses by using probes from a number of rhinoviruses. For such detailed studies it would be essential to quantitate more accurately the hybridization signals and standardize the virus titres (or even better, the quantity of viral RNAs) in the various stocks in order to obtain a precise relative assessment of the molecular relationship between the different rhinoviruses. Such studies will facilitate, perhaps more accurately, the classification of rhinoviruses into groups based on their molecular relationship rather than their serological diversity. Indeed, we hope to pursue such studies and build a more detailed picture of the relationship between members of the human rhinovirus genus. As a by-product of these studies we hope to learn more about the cross reactivities of the various cDNA probes and hence be better able to select "universal" probe(s) capable of detecting many different rhinovirus serotypes and "specific" probe(s) capable of detecting specific serotypes or groups of serotypes.

We have also presented evidence indicating that it would be feasible to detect rhinovirus in nasal washings. However, the sensitivity of detection will very much depend on the procedure followed for extracting the viral RNA. We have found that direct fixation with 20 × SSC—37% formaldehyde, as applied for the detection of enterovirus RNA by hybridization [Rotbart et al, 1984, 1985], appears to be superior to extraction of viral RNA using phenol/chloroform or fixation with only 20 × SSC. Perhaps some of the viral RNA is lost during the various steps of RNA extraction by the phenol/chloroform method leading to a decrease in sensitivity. We have shown previously [Al-Nakib et al, 1986] that the inclusion of inhibitors of endogenous RNase, such as VRC, is essential if viral RNA is to be detected consistently, especially when present in low concentrations, such as those found in clinical specimens. Indeed, our preliminary data on the direct detection of HRV-14 in clinical specimens, such as nasal washings from volunteers who have been challenged with HRV-14 and shown to excrete virus, show that it is imperative to include VRC routinely if rhinoviral RNA is to be detected by hybridization.

We hope to pursue further studies on the direct detection of rhinovirus RNA using cDNA:RNA hybridization procedures and to develop in time an appropriate clinical assay. We hope that this assay can then be modified to incorporate nonradioactive probes in order for it to have wider application.

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