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Genic amplification of the entire coding region of the HEF RNA segment of influenza C virus

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Summary

In order to provide an easy and powerful analysis of influenza C viral HEF RNA segment of a recent strain, a combination of reverse transcription and the polymerase chain reaction was used. We amplified the entire coding region of the HEF gene of a laboratory strain of virus called C/Johannesburg/1/66, widely used for binding and esterase activity studies as well as that of a strain isolated in 1991 (C/Paris/145/91) from a patient suffering from severe flu syndrome. The sequences we amplified were about 2 kilobases long. In this work, we show that the forward 'universal primer' Uni1, which has been used for influenza A and B viruses cDNA syntheses can also be used for influenza C virus. The PCR primers were designed to contain restriction sites to make the PCR products ready to be used for further purposes. A restriction analysis of the PCR products combined with analyses of all the human influenza C virus HEF gene sequences published so far permitted the design of sets of oligonucleotides which can prime PCR on cDNA of unknown influenza C virus for cloning.

Influenza C virus; Reverse transcription; Polymerase chain reaction; Detection; HEF protein

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Introduction

Influenza C virus (INF-C) is an RNA virus whose genome contains negative single-stranded segmented RNA. Unlike influenza A (INF-A) and B (INF-B) viruses, there are only 7 segments instead of 8 (Compans et al., 1977). The 'haemagglutinin' of INF-C, designated as HEF, exhibits 3 functions: haemagglutination, esterase activity and fusion factor (Vlasak et al., 1987; Herrler et al., 1988) and thus plays the roles of both haemagglutinin (HA) and neuraminidase of INF-A and INF-B. The trimeric form of HEF is inserted through the lipid bilayer by an anchor to the membrane protein or M protein. The specificities of binding and esterase activity make INF-C a tool to study 9(4)-O-acetylated sialoglycoproteins and gangliosides. We thus developed a technique of detection of these glycoconjugates using, as a probe, a laboratory strain of INF-C which underwent a large number of passages in eggs (Manuguerra et al., 1991). Although isolation of INF-C has been reported to be efficient using human melanoma cell lines (Nishimura et al., 1989), studying the HEF protein and its binding and enzymatic properties still remains difficult. Since investigation of attachment specificity and esterase activity cannot be carried out with a small amount of virus, i.e., without any adaptation on the egg (which can alter characteristics of the haemagglutinin (Robertson et al., 1985; 1987; 1991)), we thought it worth amplifying the entire coding region of HEF RNA segment. This was for use later for cloning, sequencing and expressing purposes. Very recently, PCR was applied to the C/JHB/1/66 strain of INF-C in order to amplify a 1397 base pair fragment of the HEF RNA segment with sequencing purposes. Two primers were used which were complementary to the sequence 28-42 of the non-coding strand and to the sequence 1425-1405 of the coding strand of the homologous strain (Szepanski et al., 1992). Little is known about INF-C compared to the 2 other types of influenza viruses in most respects including epidemiology, virulence and immunology. To date, for example, the hypotheses about the cyclical appearance of the virus and year-to-year variations are based on serological surveys and are not quite clear (Minuse et al., 1954; Troisi and Monto, 1981; Katagiri et al., 1983). The answers to this debate rely on virus detection when it circulates. For the time being, it is still assumed that INF-C causes only very mild influenza syndrome (Katagiri et al., 1983), but several recent isolations of strains such as C/Paris/145/91 strain, studied in the following experiments, are different and are consistent with the rare reports of severe respiratory diseases including pneumonia, mostly made in the 1950s (Andrews and McDonald, 1955; Librach, 1956; Gerber et al., 1952; Pead et al., 1985). These findings emphasise the need for further studies of influenza C virus. The aim of the study was to provide information for rapid processing of INF-C viral RNA which encodes the HEF protein, for detection, sequence analysis and protein expression.

Material and Methods

Virus isolation, propagation and antigenic characterisation

Clinical specimens were obtained during the 1990 to 1991 winter season, through the epidemiological system of surveillance of influenza set up in France by the two National Reference Centres for Influenza, the 'GROGs national network' (Hannoun et al., 1989). A strain of influenza C virus was isolated in the laboratory from a throat swab sampled from a 46-year-old woman. She suffered from intense headache and high fever (39.5°C). Sampling was carried out only 12 h after the onset of the flu syndrome. The specimen was tested for the presence of influenza A and B viruses by immunocapture (Pothier et al., 1988) and cell culture (Davies et al., 1978; Hannoun et al., 1989). The MDCK cell line used for isolation belongs to the type which expresses hardly any receptor-like molecules for INF-C (Herrler and Klenk, 1987). The specimen was negative for influenza A and B viruses as well as for Respiratory syncytial virus ('in-house' immunocapture technique and Directigen, Becton-Dickinson, USA). The clinical sample was then tested by ELISA on nitrocellulose membrane using purified polyclonal IgG prepared from a hyperimmune rabbit inoculated with C/Johannesburg/1/66 (C/JHB/1/66). It was found positive for INF-C along with 34 other specimens and then inoculated into 8-day-old embryonated eggs by the amniotic route and incubated at 35°C: amniotic and allantoic fluids were harvested three days later. After the first passage, haemagglutination was observed and virus was inoculated by allantoic route to 10-day-old embryonated eggs and harvested three days later. The virus was identified by the haemagglutination inhibition test (HI) as described previously (Dowdle et al., 1979), using chicken erythrocytes and antisera treated with receptor-destroying enzyme (Manuguerra et al., 1992). The identification name of the strain is C/Paris/145/91. The stock virus of C/Paris/145/91 used for all PCR experiments was the allantoic fluid corresponding to the first passage in eggs after isolation. The C/JHB/1/66 was used as a 'reference' strain. The strain A/Puerto Rico/8/34(H1N1) [A/PR/8/34] was included in our work as a control.

Reverse transcription and amplification of the HEF gene

Infectious allantoic fluid (first passage after isolation) (200 μl) was treated with 50 μg/ml proteinase K in 10 mM-Tris-HCl pH 7.5, 5 mM EDTA, 0.5% SDS at 37°C for 30 min. Samples were extracted once with phenol/chloroform and twice with chloroform, and nucleic acid was precipitated using ethanol with glycogen as carrier. Precipitated nucleic acid was resuspended for reverse transcription in 10 μl of 100 mM Tris-HCl pH 8.3, 10 mM $MgCl_2$, 140 mM-KCl, 10 mM-DTT, 400 μM-dNTPs and 2–3 pmol primer Uni1 (AGC AAA AGC AGG) (Robertson, 1979), specific for all segments of influenza A viruses, or A/5/1 (AAA GCA GGG GAA AAT AAA AAC AAC C) (Robertson et al., 1991) or C/10/1 (AGG GGK TTA ATA ATG TTT TTC TCA T) [K = T or

G], which are respectively specific for the 3′ end of the viral HA and HEF RNA segments. Avian myeloblastosis virus reverse transcriptase (7.5 units (U); Life Sciences Inc.) was added, and the reaction was incubated at 42°C for 60 min and terminated by boiling for 1 min.

The cDNAs corresponding to the HA1 coding region of HA RNA segment of A/PR/8/34 and the cDNA of the whole coding region of HEF RNA segment of C/JHB/1/66 and C/Paris/145/91 were amplified enzymically in a 100-μl PCR containing 10 mM-Tris-HCl pH 8.3, 3 mM $MgCl_2$, 50 mM-KCl, 0.01% (w/v) gelatin, 0.1 μM primers, 200 μM-dNTPs and 2.5U Taq polymerase (Life Sciences Inc.), overlaid with paraffin oil and subject to 30 cycles of 94°C for 0.48 min, 45°C for 0.48 min and 72°C for 1.5 min (Saiki et al., 1988). The 30 cycles were preceded by a step reaction at 94°C for 10 min and followed by a terminating polymerisation step at 72°C for 10 min. The primers used for amplification were Uni1, A/5/1, A/1117/2 (ATC ATT CCA GTC CAT CCC CCT TCA AT) (Robertson et al., 1991), C/10/1, C/2046/2 (TTT ATA AAR CTG TAC AAA ATA TTG) [R = A or G], C/1918/2 (TAA NNN NNN GCT TCA TCA CCA GTA AAA AGG), C/2032/2 (CAA AAT NNN NNN YAA YAY ATT ATC CAT) [Y = C or T]. The EnzC/1918/2 and EnzC/2032/2 primers have a built-in restriction site (underlined, where N can be any nucleotide), the designation of these primers is preceded by the corresponding acronym of enzyme. The *Xba*IC/2032/2 and *Sal*IC/2032/2 (where NNN NNN are T″CT AGA and G″TCGAC respectively) were used for cloning purposes, the former was chosen for pSV-SPORTI, the latter for bacteriophage M13mp19, (where the *Sma*I site is too close to *Xba*I). The sequence coding for an anchorless form of HEF was amplified for future protein expression in pSV-SPORTI using the *Hin*dIIIC/1918/2 (where NNN NNN is A″AG CTT) as well as *Xba*IC/1918/2 (where NNN NNN is T″CT AGA) to amplify the portion of the HEF segment corresponding to the extramembrane part of HEF protein. After amplification, 10 μl of each reaction were analysed by agarose gel electrophoresis and the DNA was visualised by ethidium bromide. All extractions, cDNA syntheses and amplifications were accompanied by controls in which water replaced the sampled being analysed, including a control which was co-processed through every manipulation. All pre-amplification manipulations were carried out in a room separate from that used for post-amplification work.

DNA purification, digestion by restriction enzymes and sequence analyses

Following amplification, the DNA was loaded on a 1% agarose gel for electrophoresis and the specifically amplified band of DNA was cut from the gel under UV light. The piece of agarose was transferred into a cell for electroelution with TBE (0.09 M Tris-borate; 0.002 M EDTA, pH = 8) 0.1 × by a 2-mA electric current. DNA was then precipitated with ethanol and sodium acetate (200 mM final concentration), washed and dried. The occurrence of the following restriction sites was studied on purified PCR products: *Hin*dIII, *Sac*I,

*Sal*I, *Sma*I and *Xba*I (enzymes were purchased from Boehringer Mannheim, Germany).

Analyses were carried out on all the sequences of human INF-C HEF cDNAs published so far obtained from Genbank. Their accession numbers as well as their corresponding references are indicated in Tables 1 and 5. Search of restriction sites within the cDNA sequences and the calculations of DNA fragment sizes (Schaffer and Sederoff, 1981) were computer-assisted by Seqaid version 3.7, a software created by D.D. Rhoads and D.J. Roufa (Kansas State University, USA).

Results

The first isolate of influenza C virus in France (C/Paris/145/91) since the isolation of C/Paris/1/67 was obtained from an adult during the 1990 to 1991 winter season and was studied. All manipulations were carried out with A/PR/8/34(H1N1), C/JHB/1/66 and C/Paris/145/91. All experiments on influenza C virions were carried out with freshly harvested virus immediately or after having been frozen at −80°C upon harvesting; otherwise most results were negative. The HA titre of each stock of virus used was 32 or diluted to this titre. Initially, we used the universal primer Uni1 which can be utilised to synthesise cDNA from all segments of influenza A and B viruses. The full length of C/JHB/1/66 HEF RNA segment is 2073 nucleotides (Pfeiffer and Compans, 1984) and that of C/California/78 is 2071 (Nakada et al., 1984).

The sequences of the primers specific for the HEF segment of INF-C were based on C/JHB/1/66 and C/California/78. Using Seqaid ver 3.7, analyses were carried out to calculate the number of mismatches between the primers and the sequences of all available HEF genes from human INF-C (Table 1). The positions of the first nucleotide of each primer within the targeted gene are indicated. The numbering of each strain starts with the first nucleotide sequenced as no. 1. As shown in Table 1, most strains have not been sequenced from the very first nucleotide at the 3′ end of the gene. As expected, the extent of mismatch (number of mismatches/length of the respective primer) between A/5/1, A/1117/2 and HEF gene sequences was very high, varying from 40 to 83%. On the contrary, EnzC/1918/2 completely matched with all the HEF gene sequences, the 7 or 8 mismatches being due to the insertion of 2 stop codons and 1 restriction site. EnzC/2032/2 matched perfectly at the expected position with 7 strains out of the 11 analysed. However, the score of mismatches was high, between 33% and 52% (depending on the restriction site built into the primer), with 4 strains C/Nara/82, C/Mississipi/80, C/Kyoto/41/82 and C/Hyogo/83. C/2046/2 matched perfectly and at the expected position with 8 strains including the 7 strains mentioned above for EnzC/2032/2 plus C/Mississipi/80 (Table 1). The score of mismatches was high (46%) with C/Nara/82, C/Kyoto/41/82 and C/Hyogo/83.

As shown in Figure 1, lane 5 and Table 2, the cDNAs RNA segments of C/

TABLE 1

Mismatches between primers and HEF genes

All the HEF genes of human INF-C sequenced so far were obtained from Genbank. Their names are followed by their accession number and the publication reference. The size of HEF gene sequences are indicated. NSR means non-sequenced region. NSR indicates that the sequence of the 3' non-coding region of the genome segment is not available and that the comparison of the position of the match of the different primers should take this into consideration. NA means not available. The names of the primers are those used in Material and Methods. Nt means nucleotide. The length of each primer is shown. A search of matching for each primer has been performed on whole genes with Seqaid ver 3.7. In each box, the upper number indicates the location of the match of the first nucleotide of the primer within the HEF gene sequence. The lower figure is the number of mismatches between the primer and the viral gene. The first column under EnzC/2032/2 and EnzC/1918/2 shows the number of mismatches excluding the built-in restriction site.

Primers:	HEF	Uni1	A/5/1	A/1117/2	C/10/1	C/2046/2	EnzC/2032/2			EnzC/1918/2		
Strains:	Gene size	12 Nt at the 3' end	25 Nt at the 3' end	27 Nt in the middle	25 Nt at the 3' end	24 Nt at the 5' end	27 Nt at the 5' end	*Xba*I 27 Nt at the 5' end	*Sal*I 27 Nt at the 5' end	30 Nt at the 5' end	*Xba*I 30 Nt at the 5' end	*Hin*dIII 30 Nt at the 5' end
C/Taylor/ 1233/47	2015	NA	1	1781	NA	1967	1950	1950	1950	1833	1833	1833
(sequence; A.N: M11637 Buonaugurio et al., 1985)	+NSR		24	13		0	0	5	2	3	7	8
C/Nara/82	1923	NA	1	1782	NA	1856	1704	1704	1704	1825	1825	1825
(sequence; A.N: M25362 JO4363 Adachi et al., 1989)	+NSR		20	12		11	9	14	14	3	7	8
C/Mississipi/80	2010	NA	1	1778	NA	1964	1709	1709	1709	1830	1830	1830
(sequence; A.N: M11640; Buonaugurio et al., 1985)	+NSR		20	13		3	9	14	14	3	7	8
C/Yamagata/10/83	2015	NA	1	1965	NA	1967	1950	1950	1950	1833	1833	1833
(sequence; A.N: M11640: Buonaugurio et al., 1985)	+NSR		20	17		1	0	5	2	3	7	8

Primers:	HEF	Uni1	A/5/1	A/1117/2	C/10/1	C/2046/2	EnzC/2032/2			EnzC/1918/2		
Strains:	Gene size	12 Nt at the 3′ end	25 Nt at the 3′ end	27 Nt in the middle	25 Nt at the 3′ end	24 Nt at the 5′ end	27 Nt at the 5′ end	*Xba*I 27 Nt at the 5′ end	*Sal*I 27 Nt at the 5′ end	30 Nt at the 5′ end	*Xba*I 30 Nt at the 5′ end	*Hin*dIII 30 Nt at the 5′ end
C/California/78 (sequence; A.N: KO1689; Nakada et al., 1984)	*2071*	*1* *0*	*1* *12*	*1606* *14*	*10* *0*	*2022* *0*	*2005* *0*	*2005* *5*	*2005* *2*	*1888* *3*	*1888* *7*	*1888* *8*
C/Kyoto/41/82 (sequence; A.N: M25361 JO4363; Adachi et al., 1989)	1923 +NSR	NA	1 20	1782 12	NA	1856 11	1704 9	1704 14	1704 14	1825 3	1825 7	1825 8
C/England/892/83 (sequence; A.N: M11642; Buonaugurio et al., 1985)	2015 +NSR	NA	1 20	1781 13	NA	1967 0	1950 0	1950 5	1950 2	1833 3	1833 7	1833 8
C/Hyogo/1/83 (sequence; A.N: M25363 JO4363; Adachi et al., 1989)	1923 +NSR	NA	1 19	1782 12	NA	1856 11	1704 9	1704 14	1704 14	1825 3	1825 7	1825 8
C/Ann Arbor/1/50 (sequence; A.N: M11638; Buonaugurio et al., 1985)	2015 +NSR	NA	1 20	1781 13	NA	1967 0	1950 0	1950 5	1950 2	1833 3	1833 7	1833 8
C/Great Lakes /1167/54 (sequence; A.N: M11639; Buonaugurio et al., 1985)	2014 +NSR	NA	1 20	1781 14	NA	1967 0	1950 0	1950 5	1950 2	1833 3	1833 7	1833 8

Primers:	HEF	Uni1	A/5/1	A/1117/2	C/10/1	C/2046/2	EnzC/2032/2			EnzC/1918/2		
Strains:	Gene size	12 Nt at the 3′ end	25 Nt at the 3′ end	27 Nt in the middle	25 Nt at the 3′ end	24 Nt at the 5′ end	27 Nt at the 5′ end	*Xba*I 27 Nt at the 5′ end	*Sal*I 27 Nt at the 5′ end	30 Nt at the 5′ end	*Xba*I 30 Nt at the 5′ end	*Hin*dIII 30 Nt at the 5′ end
C/JHB/1/66 (sequence; A.N: M17868; Pfeifer and Compans, 1984)	*2073*	*1* *1*	*5* *13*	*2000* *18*	*10* *0*	*2025* *0*	*2008* *0*	*2008* *5*	*2008* *2*	*1891* *3*	*1891* *7*	*1891* *8*

NSR: non-sequenced region.
NA: not available.
The 3′ and 5′ ends refer to the respective ends of the HEF genome segment.

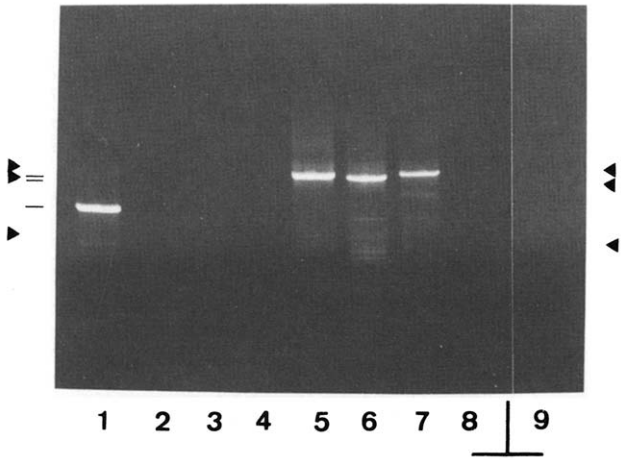

Fig. 1. PCR on cDNAs synthesised with Uni1. After reverse transcription with Uni1 as described in Material and Methods, cDNAs were submitted to PCR using different primers and various combinations of them as indicated in Table 3 for each lane. The target gene was segment 4 of the strains A/PR/8/34, C/JHB/1/66 and C/Paris/145/91. The position of the bands are shown in the margin. The molecular weights indicated by arrow heads in the margins are 2320 2020 and 560 base pairs.

TABLE 2

Sizes of PCR products

Expected and observed sizes of PCR products are summarised. The sizes of the expected fragments were calculated according to the published sequences of C/JHB/1/66 (Pfeiffer and Compans, 1984) and A/PR/8/34 (Winter et al., 1981).

Target gene	Strain	cDNA synthesis primer	PCR pair of primers	Expected size of PCR product (bp)	Observed size of PCR product (bp)	sd (bp)
HEF	C/JHB/1/66	Uni1	C/10/1-C/2046/2	2036	2035	20
HEF	C/JHB/1/66	Uni1	C/10/1-*Hin*dIIIC/1918/2	1908	1903	27
HEF	C/JHB/1/66	C/10/1	C/10/1-C/2046/2	2036	2029	12.5
HEF	C/Paris/145/91	C/10/1	C/10/1-C/2046/2	NA	2035	20
H1	A/PR/8/34	Uni1	A/5/1-A/1117/2	1112	1129	30

JHB/1/66, which had been synthesised with the 'influenza universal primer' Uni1 were submitted to amplification with the C/10/1-C/2046/2 pair of primers specific for segment 4 of influenza C virus, and a band of 2035 base pairs (bp) (sd = 20 bp) (Table 2) was detectable on a 1% agarose gel containing ethidium bromide after electrophoresis. A band of 1903 bp (sd = 27 bp) was detectable when total cDNA of C/JHB/1/66 was submitted to amplification with the Uni1-*Hin*dIIIC/1918/2 pair of primers (Table 3) (Fig. 1, lane 6). A band of 1908 bp was expected (Table 2). As a positive reaction control (Fig. 1, lane 1), A/PR/8/34 RNA segments were transcribed into cDNAs with Uni1 and the cDNAs were then amplified with the A/5/1 and A/1117/2 primers specific for the HA1

TABLE 3

Combinations of PCR primers used to amplify cDNAs synthesised with Uni1
The numbers of the lanes correspond to the numbers of the lanes of the gel shown in Fig. 1. For each lane, the combination of PCR primers are indicated by + and − signs.

Lanes:	1	2	3	4	5	6	7	8	9
PCR primers:									
A/5/1	+	−	+	+	−	−	−	+	−
A/1117/2	+	−	+	+	−	−	−	+	−
C/10/1	−	+	−	−	+	+	+	−	+
C/2046/2	−	+	−	−	+	−	+	−	+
*Hin*dIII									
C/1918/2	−	−	−	−	−	+	−	−	−
target:									
A/PR/8/34	+	+	−	−	−	−	−	−	−
C/JHB/1/66	−	−	+	−	+	+	−	−	−
C/Paris/145	−	−	−	+	−	−	+	−	−

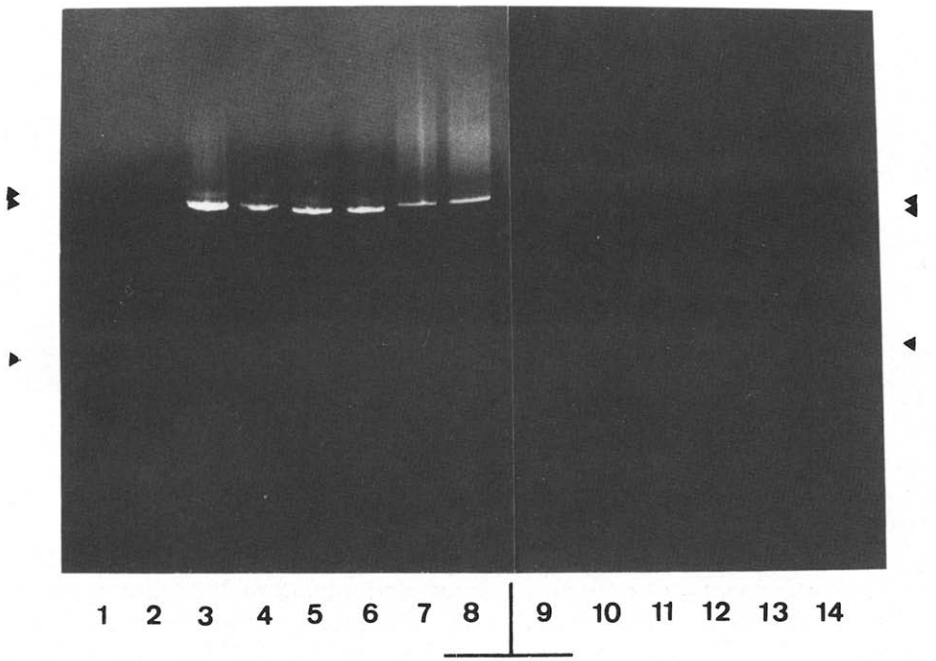

Fig. 2. PCR on cDNAs synthesised with primers specific for segment 4 of influenza A or C viruses. Reverse transcription was performed with A/5/1 or C/10/1 specific for the HA or the HEF RNA segment of influenza A and C viruses respectively (as described in Material and Methods). cDNAs were then submitted to PCR using different primers and various combinations of them as indicated in Table 4 for each lane. The target gene was segment 4 of the strains A/PR/8/34, C/JHB/1/66 and C/Paris/145/91. The molecular weights indicated by arrowheads in the margins are 2320, 2020 and 560 base pairs.

coding region of A(H1N1) segment 4 (Table 3). A band of 1129 bp (sd = 30 bp) was detectable against a theoretical size of 1112 bp (Table 2). On the contrary, these A/PR/8/34 cDNAs were not amplified when the pair of primers specific for influenza C HEF RNA segment was used (Fig. 1, lane 2) (Table 3). A band around 1000 bp was not detectable when INF-C cDNAs were first synthesised with Uni1 and then amplified with the A/5/1-A/1117/2 pair of primers (Fig. 1, lanes 3 and 4) (Table 3). All negative controls were negative as shown in Fig. 1, lanes 8 and 9.

The synthesis of cDNA was also carried out with the C/10/1 primer designed for the 4th segment of influenza C virus genome (Table 4). No amplification could be detected when A/PR/8/34 cDNA primed with C/10/1 was submitted to PCR with the A/5/1-A/1117/2 pair of primers (Fig. 2, lane 1) (Table 4). As expected no band was visualised when the cDNA of HA RNA segment of A/PR/8/34 was amplified with the C/10/1-C/2046/2 pair of primers (Fig. 2, lane 2) (Table 4). On the contrary, the cDNA of the HEF RNA of C/JHB/1/66 and C/Paris/145/91 were both amplified when the reaction was primed with C/10/1-C/2046/2 (Fig. 2, lanes 3 and 4) (Table 4). All negative controls were negative as shown in Fig. 2, lanes 9 to 14 (Table 4).

When 30 cycles of 94°C for 0.48 min, 55°C for 0.48 min and 72°C for 1.5 min were applied, amplification of INF-C was not observed, whereas amplification

TABLE 4

Combinations of reverse transcription and PCR primers used to amplify HEF cDNAs synthesised with A/5/1 or C/10/1

The numbers of the lanes correspond to the numbers of the lanes of the gel shown in Fig. 2. For each lane, the combination of reverse transcription and PCR primers are indicated by + and − signs.

Lanes:	1	2	3	4	5	6	7	8	9	10	11	12	13	14
RT primer:														
A/5/1	−	+	−	−	−	−	−	−	−	−	−	−	−	+
C/10/1	+	−	+	+	+	+	+	+	+	+	+	+	+	−
PCR primers:														
A/5/1	+	−	−	−	−	−	−	−	−	−	−	−	−	+
A/1117/2	+	−	−	−	−	−	−	−	−	−	−	−	−	+
C/10/1	−	+	+	+	+	+	+	+	+	+	+	+	+	−
C/2046/2	−	+	+	+	−	−	−	−	+	−	−	−	−	−
*Hin*dIII														
C/1918/2	−	−	−	−	+	−	−	−	−	+	−	−	−	−
*Xba*I														
C/1918/2	−	−	−	−	−	+	−	−	−	−	+	−	−	−
*Xba*I														
C/2032/2	−	−	−	−	−	−	+	−	−	−	−	+	−	−
*Sal*I														
C/2032/2	−	−	−	−	−	−	−	+	−	−	−	−	+	−
Target:														
A/PR/8/34	+	+	−	−	−	−	−	−	−	−	−	−	−	−
C/JHB/1/66	−	−	+	−	−	+	+	+	−	−	−	−	−	−
C/Paris/145	−	−	−	+	+	−	−	−	−	−	−	−	−	−

TABLE 5

Restriction sites analysis of HEF genes
The restriction sites analysis has been computer-assisted with Seqaid ver 3.7 and performed for all the sequences of the HEF gene of human INF-C published so far. They were obtained from Genbank and their names are followed by their accession number and the references of publication. The top figure represents the number of the respective restriction site followed by their position within the gene. The bottom figures represent the sizes of the fragments.

	*Hin*dIII	*Sal*I	*Sma*I	*Xba*I
C/Taylor/1233/47 (sequence; A.N: M11637; Buonaugurio et al., 1985)	2 sites: 994/1570 Fragment sizes: 445/576/994	0 site.	0 site.	0 site.
C/Nara/82 (sequence; A.N: M25362 JO4363; Adachi et al., 1989)	2 sites: 986/1562 Fragment sizes 361/576/986	0 site.	0 site.	0 site.
C/Mississipi/80 (sequence; A.N: M11640; Buonaugurio et al., 1985)	2 sites: 1052/1628 Fragment sizes: 445/576/1052	0 site.	0 site.	0 site.
C/Yamagata/10/83 (sequence; A.N: M11640; Buonaugurio et al., 1985)	1 site: 994 Fragment sizes: 994/1021	0 site.	0 site.	0 site.
C/California/78 (sequence; A.N: KO1689; Nakada et al., 1984)	0 site.	0 site.	0 site.	0 site.
C/Kyoto/41/82 (sequence; A.N: M25361 JO4363; Adachi et al., 1989)	2 sites: 986/1562 Fragment sizes 361/576/986	0 site.	0 site.	0 site.
C/England/892/83 (sequence; A.N: M11642; Buonaugurio et al., 1985)	0 site.	0 site.	0 site.	0 site.
C/Hyogo/1/83 (sequence; A.N: M25363 JO4363; Adachi et al., 1989)	2 sites: 986/1562 Fragment sizes: 361/576/986	0 site.	0 site.	0 site.
C/Ann Arbor/1/50 (sequence; A.N: M11638; Buonaugurio et al., 1985)	1 site: 994 Fragment sizes: 994/1021	0 site.	0 site.	0 site.
C/Great Lakes/1167/54 (sequence; A.N: M11639; Buonaugurio et al., 1985)	1 site: 1570 Fragment sizes: 444/1570	0 site.	0 site.	0 site.
C/JHB/1/66 (sequence; A.N: M17868; Pfeifer and Compans, 1984)	2 sites: 1052/1628 Fragment sizes: 445/576/1052	0 site.	0 site.	0 site.
C/JHB/1/66 PCR product	2 sites: Fragment sizes: 367/559/1069 (sd = 12.5)	0 site.	0 site.	0 site.
C/Paris/145/91 PCR product	2 sites: Fragment sizes: 362/561/1101 (sd = 20)	0 site.	0 site.	0 site.

of A/PR/8/34 was successful (data not shown). These results led to using the conditions described in Material and Methods. According to the results in Figs. 1 and 2, specificity was not affected by a lower temperature of annealing (45°C), although rare extraneous bands were present.

The products of amplification of the full length HEF cDNA (from position 10 to 2046) obtained for C/Paris/145/91 can be used for direct sequencing. The products of the PCR primed with backward primers into which restriction sites had been built, such as *Hin*dIIIC/1918/2, *Xba*IC/1918/2, *Xba*IC/2032/2 and *Sal*IC/2032/2 (Table 4), were used to prepare DNA fragments (Fig. 2, lanes 5–8) for cloning purposes in the sequencing vector: bacteriophage M13mp19 (Boehringer Mannheim, Germany) and also in the shuttle expression vector: plasmide pSV-SPORTI (Gibco/BRL, USA). The occurrence of *Hin*dIII, *Sac*I, *Sal*I, *Sma*I and *Xba*I restriction sites was assessed for all the sequences of INF-C HEF cDNA published so far, obtained from Genbank, and also in purified PCR products. As shown in Table 5, the existence of a *Hin*dIII restriction site, which was predictable from the published sequence of C/JHB/1/66, was confirmed for the PCR product. Occurrence of *Hin*dIII restriction sites varies from strain to strain. As shown in Table 5, the number of sites varies from 0 to 2. The strains C/California/78 and C/England/892/83 have no *Hin*dIII site, whereas C/Taylor/1233/47, C/Nara/82, C/Mississipi/80, C/Kyoto/41/82 as well as C/JHB/1/66 have 2 sites and C/Yamagata/10/83, C/Ann Arbor/1/50, C/Great Lake/1167/54 have 1 site. The positions of *Hin*dIII restriction sites are 986, 994 or 1052 and/or 1562, 1570 or 1628. When cDNA of C/JHB/1/66 was submitted to PCR with C/10/1-C/2046/2, the length of the undigested PCR product was 2029 bp (sd = 12.5 bp), which is in accordance with the sequence data (Table 2). After digestion, 3 bands were generated: 1069 bp (sd = 12.5 bp), 559 bp (sd = 12.5 bp) and 367 bp (sd = 12.5 bp) instead of 1052 bp, 576 bp and 445 bp we expected as indicated in Table 5. The electrophoreses were carried out on a submarine mini-gel and this may account for a lack of accuracy of measurement. As shown in Fig. 2, lane 4, when HEF cDNA from C/Paris/145/91 was the template for PCR (Table 4), the length of the undigested product was 2035 bp (sd = 20 bp) (Table 2). After digestion by *Hin*dIII, there were three bands of 1101 bp (sd = 20 bp), 561 bp (sd = 20 bp) and 362 bp (sd = 20 bp) as shown in Table 5, the total of which being 2024 bp (± 60 bp). These findings are close to those for C/Hyogo/1/83, C/Kyoto/41/82 and C/Nara/82 (all Japanese strains) by analysis of their sequence as shown in Table 5. No *Sac*I, *Sal*I, *Sma*I and *Xba*I restriction sites existed in the full length HEF gene of C/JHB/1/66 as expected from the sequence as well as in any HEF gene of any INF-C sequenced so far including C/Paris/145/91 (Table 5). As a consequence, the DNA amplified with the C/10/1-*Xba*IC/1918/2, C/10/1-*Xba*IC/2032/2 or C/10/1-*Sal*IC/2032/2 pairs of primers can be used for oriented cloning experiments in the polycloning sites of bacteriophage M13mp19 and pSV-SPORTI with little risk of encountering undesired restriction sites in an unknown strain. Digestions of bacteriophage M13mp19 and pSV-SPORTI with *Sma*I and *Xba*I can be carried out in the same incubation buffer and

generate one blunt end and one cohesive end in the correct orientation. The DNA amplified with the C/10/1-*Sal*IC/2032/2 pair of primers (Fig. 2, lanes 6, 7, 8) can also be cloned in both types of vectors using *Sma*I and *Sal*I for digestions. The DNA amplified with pairs of primers containing *Hin*dIII restriction sites such as *Hin*dIIIC/1918/2 cannot be used for cloning because of internal *Hin*dIII sites in the gene of interest in C/Paris/145/91.

Discussion

There has been little interest in INF-C epidemiology and genetics and there is to date only one report concerning genic enzymatic amplification or PCR applied to the HEF RNA this type of virus, but it was applied to a partial amplification of the gene of the laboratory strain C/JHB/1/66 (Szepanski et al., 1992). As the virus itself requires specific means and efforts for isolation or detection, we believe that PCR can provide an easily available method and useful information about the real importance of INF-C as a pathogen. This would be an indispensable complement to sero-epidemiological surveys which have shown that INF-C is an agent of a widespread infection (Minuse et al., 1954; Dyke et al., 1980; O'Callaghan et al., 1980; Andrews and McDonald, 1955; Gerth et al., 1975; Jennings et al., 1968; Homma et al., 1982; Nishimura et al., 1987; Manuguerra et al., 1992). The strain recently isolated in our laboratory (C/Paris/145/91) is a prime example that INF-C can cause a severe flu syndrome. This contradicts views widely held about the mild clinical expression of this virus based on a single study (Katagiri et al., 1983) in spite of rare reports dealing with acute and severe illness caused by INF-C (Andrews and McDonald, 1955; Librach, 1956; Gerber et al., 1952; Pead et al., 1985). Detection alone is not sufficient and further detailed analyses such as sequence determination should be carried out. Moreover, the binding and enzymatic specificities of the HEF protein make INF-C a unique and powerful tool to study 9(4)-O-acetylated sialoglycoconjugates, much appreciated by biochemists. PCR experiments as described here carried out on the full length HEF gene enables direct sequencing or cloning of vRNA for expression experiments. Most binding properties and enzymatic characteristics of the esterase activity of INF-C, described so far have been assessed on old strains whose passage history is unclear. Recent work (Robertson et al., 1985, 1987, 1991) demonstrates that this property is very dependent on passage history, especially in eggs. We lack information about recent strains which have a very short passage history. PCR could help to increase our knowledge about the binding capacity of 'wild' INF-C, about the specificity of esterase activity and its possible role in pathogenesis. Isolation of INF-C can be carried out with high yields of virus on only a limited number of cell lines which display a sufficient amount of 9(4)-O-acetylated sialic acids on their surface, e.g. human melanoma cell lines (Nishimura et al., 1989; unpublished personal data). Human cancer cell lines are not usually used for isolation of INF-A and INF-

B. The identification by haemagglutination inhibition test (HI) or by ELISA on membrane or on antibody coated plates relies on strict antibody specificity. According to our experience, and as anticipated, due to similar features between some coronaviruses and INF-C (Vlasak et al., 1988), there is a wide serological cross-reactivity between INF-C and respiratory human coronaviruses with some monoclonal antibodies. This may be due to the large homology of amino-acid sequence of INF-C HEF and a coronavirus glycoprotein such as the HEX protein of mouse hepatitis virus (Luytjes et al., 1988).

Our results show that INF-C RNA can be transcribed reversibly into cDNA using the universal primer for INF-A and INF-B: Uni1 (Robertson et al., 1979; Desselberger et al., 1980). The sequence of this primer is based on the conserved sequence of the short 3′ non-coding region of the genomic segments of influenza A (Robertson et al., 1979). Moreover, using universal primer Uni1 enables the conservation of all viral genes.

Since the non-coding sequence at the 3′ end of all segments of INF-A, INF-B and INF-C is closely conserved, (Desselberger et al., 1980), it is sensible to assume that Uni1 may match the 3′ end of the HEF gene of any strain of INF-C. As shown in Table 1, most of INF-C strains, whose HEF gene sequence has been analysed, can be aligned without any gaps and with a very low score of mismatch with all the primers specific for the 5′ end of HEF gene. Three strains C/Nara/82, C/Kyoto/41/2 and C/Hyogo/83 can match only with EnzC/1918/2. C/Mississipi/80 matches with EnzC/1918/2 and C/2046/2. C/Taylor/1233/47, C/Yamagata/10/83, C/England/892/83, C/Ann Arbor/1/50, C/Great-Lakes/1167/54 and of course C/JHB/1/66, C/California/78 and C/Paris/145/91 matched perfectly with all the 11 primers mentioned above. These results indicate that the sets of primers for reverse transcription and PCR used in this work can be used for any strain of INF-C and among them particularly Uni1-EnzC/1918/2 and C/10/1-EnzC/1918/2.

The results also show the type specificity of cDNA synthesis when we utilised a primer which overlaps the signal peptide coding region. It is specific for the HA RNA segment of INF-A or for the HEF RNA segment of INF-C. The PCR, which followed reverse transcription, was also type-specific since cDNA of A/PR/8/34 as well as C/JHB/1/66 and C/Paris/145/91 synthesised with Uni1 were not amplified with primers specific for INF-C HEF gene or INF-A(H1N1) HA gene respectively. No cross-amplification was observed when the cDNA of A/PR/8/34 or C/JHB/1/66 and C/Paris/145/91 were synthesised with a primer specific for the 4th segment of INF-A and INF-C respectively. Since the universal primer Uni1 can be used to prime the synthesis of cDNA of any influenza virus, a unique reverse transcription mixture with a single primer is sufficient. As PCR is type-specific (according to the above results) and even sub-type specific (Robertson et al., in press), a cocktail of pairs of primers specific for A(H1N1), A(H3N2), B and C viruses can potentially be used for synthesis of fragments with different sizes. This would allow detection and identification of any influenza A, B but also C virus in a single test and would be particularly useful for epidemiological studies.

The efficacy of reverse transcription and PCR was lower for the full length HEF gene than for the 1000 bp fragment of A/PR/8/34 (results not shown). The reason may be due to size and thus to a longer synthesis of the HEF target which the Taq polymerase could not complete as efficiently. Since the aim of this work was not only detection, the length of the amplified cDNA was deliberately not shortened. In order to express a soluble form of HEF, thus easily available in a eukaryotic expression system, we also amplified the part of the sequence coding for an anchorless protein. The target was the region of the HEF gene which extends from the signal peptide to the beginning of the hydrophobic stretch that transverses the lipid bilayer of the envelope. The sequence of the primers have been determined according to that of C/JHB/1/66 (Pfeiffer and Compans, 1984) and C/California/78 (Nakada et al., 1984). Two stop codons have been built into these primers. The expected protein would lack the proximal stretch of hydrophobic residues and the anchor to the membrane protein.

The choice of the restriction sites inserted into some primers was based upon all the sequences of human INF-C HEF genes. Only 11 have been published so far, although interest in INF-C has progressively increased during the past 6 years. This shows the necessity of developing studies about this virus. The number of INF-C strains isolated during the winter 1990–1991 (35 detections, 4 isolations), which is the highest ever obtained in our laboratory, contrasts with that of INF-B and A strains isolated during the same period of time: 204 (202 INF-B, 2 INF-A(H3N2)) (unpublished results). Unfortunately we could not perform any experiment directly on clinical samples, because of shortage of original virus material. For this reason we worked with stock virus which had undergone only 2 passages in eggs. We chose 4 restriction sites which are not present in the HEF genes already sequenced. They are present and correctly oriented in the cloning and expression vectors we intend to use. The occurrence of the *Hin*dIII restriction site was studied because it is one of the variable sites in INF-C strains. The primers published in this report (*Xba*IC/2032/2, *Xba*IC/1918/2, *Sal*IC/2032/2, *Sal*IC/1918/2) are likely to be suitable for the amplification and cloning of any INF-C. The PCR fragments we obtained are ready for cloning in either of the 2 vectors mentioned above, for multiple use.

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