UNIVERSITÉ DU QUÉBEC

THESIS

SUBMITTED TO

THE INSTITUT ARMAND-FRAPPIER

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF MASTER OF SCIENCE

IN VIROLOGY AND IMMUNOLOGY

BY

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THE DETECTION OF THE NUCLEIC ACID OF HUMAN CORONAVIRUSES 229E AND OC43 IN TISSUE SPECIMENS USING THE REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

AUGUST 1992

To the all too great portion of humanity which has yet to benefit substantially from the fruits of scientific endeavor.

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LIST OF ABBREVIATIONS

ATP	: Adenosine 5'-triphosphate
BCV	: Bovine coronavirus
CCV	: Canine coronavirus
CNS	: Central nervous system
CTP	: Cytidine triphosphate
DEPC	: Diethylpyrocarbonate
DNA	: Deoxyribonucleic acid
DNase	: Deoxyribonuclease
DTT	: Dithiothreitol
EDTA	: Ethylenediaminetetraacetic acid
FECV	: Feline enteric coronavirus
FIPV	: Feline infectious peritonitis virus
HCV	: Human coronavirus
HECV	: Human enteric coronavirus
HEV	: Hemagglutinating encephalomyelitis virus
IBV	: Infectious bronchitis virus
IPTG	: Isopropyl ß-D-thiogalactopyranoside
L132	: Human embryonic lung fibroblasts
MBP	: Myelin basic protein
MHV	: Murine hepatitis virus
mini-prep	: mini-preparation
MOPS	: 3-[N-morpholino] propanesulfonic acid
MS	: Multiple sclerosis

dNTP	: Deoxyribonucleoside triphosphate
ORF	: Open reading frame
PBL	: Peripheral blood lymphocyte
PCI	: Phenol:chloroform:isoamyl alcohol 24:24:1
PCR	: Polymerase chain reaction
PEG	: Polyethylene glycol
PRCV	: Porcine respiratory coronavirus
pg	: Picogram
RbCV	: Rabbit coronavirus
RER	: Rough endoplasmic reticulum
RNA	: Ribonucleic acid
mRNA	: messenger RNA
rRNA	: ribosomal RNA
RT-PCR	: Reverse transcription-polymerase chain reaction
SDS	: Sodium dodecyl sulfate
Taq	: *Thermophilus aquaticus*
TCV	: Turkey coronavirus
TGEV	: Transmissible gastroenteritis virus
x-gal	: 5-Bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

SOMMMAIRE

Sur la base d'études épidémiologiques, on soupçonne un facteur environnemental, en particulier un virus, d'être à l'origine de la sclérose en plaques. Plusieurs données permettent de croire que les coronavirus humains se trouvent parmi les virus potentiellement impliqués. D'abord, des particules coronaviriformes ont été observées en microscopie électronique dans une coupe de cerveau provenant d'un patient atteint de sclérose en plaques; de plus on retrouve des anticorps anti-HCV-229E et anti-HCV-OC43 dans le liquide céphalo-rachidien d'un nombre significatif de patients atteints de cette maladie.

Le présent projet est une étude préliminaire qui visait d'abord la mise au point de la technique de la transcription inverse suivie de la réaction de polymérisation en chaîne (RT-PCR) pour la détection de l'ARN des coronavirus humains, les HCV-229E et HCV-OC43, dans les tissus humains. Par la suite, cette méthode a été appliquée à l'analyse d'échantillons cliniques provenant de diverses sources. Pour s'assurer de la bonne préservation de l'ARN dans les échantillons, nous avons vérifié la qualité de celui-ci en effectuant la détection par RT-PCR d'ARNm cellulaire, soit l'ARNm codant la Γ-actine humaine ou la protéine basique de la myéline, selon le cas. Cette première étape a été effectuée avec succès puisque nous

avons pu démontrer la présence d'acide nucléique du coronavirus humain souche 229E dans certains prélèvements et nous avons estimé à 60,000 le nombre de molécules détectables par une paire d'amorces. Nous avons procédé à l'analyse des lymphocytes périphériques de sujets sains pour la présence de l'ARN des deux virus en question et trois échantillons sur 13 se sont avérés positifs à deux reprises pour l'ARN du HCV-229E. Étant donné le petit nombre d'échantillons, nous considérons ces résultats comme très prometteurs par rapport à la caractérisation du tropisme du HCV-229E. L'absence d'ARN du HCV-OC43 dans les mêmes prélèvements nous suggère que ce virus n'infecte pas ces cellules, mais, comme pour le HCV-229E, un plus grand nombre de sujets doit être examiné pour confirmer ces observations. Une étude des prélèvements de tissus provenant de cerveaux de patients atteints de sclérose en plaques ainsi que des témoins a aussi été effectuée. Quatre des 21 échantillons provenant de 11 patients ont donné des résultats positifs pour l'ARN du HCV-229E lesquels ont été confirmés par des épreuves subséquentes. Aucun des onze échantillons des cerveaux témoins n'a été confirmé comme étant positif. Aucun échantillon n'a donné de résultat positif pour le HCV-OC43. Nos résultats avec les prélèvements de cerveaux soulignent l'importance de continuer à étudier la possible implication du virus HCV-229E dans des maladies neurologiques.

SUMMARY

On the basis of epidemiologic studies, an environmental agent, particularly a virus, is suspected of being the cause of multiple sclerosis. Human coronaviruses number among the potential etiologic agents due to several observations. Coronavirus-like particles were observed by the electron microscope in a brain section from a patient with multiple sclerosis and antibody to human coronaviruses 229E and OC43 are detectable in the cerebrospinal fluid of a significant proportion of MS patients.

The present project involved the setting up of the reverse transcription-polymerase chain reaction technique for the detection of human coronaviruses, HCV-229E and HCV-OC43, and a preliminary study of human tissue specimens, including CNS tissues from multiple sclerosis patients, for the presence of the nucleic acid of the two viruses. The quality of the RNA extracted from archival tissues was verified using an additional RT-PCR test to detect cellular messenger RNAs in them, either human MBP RNA in white matter or Γ-actin mRNA in PBLs and in grey matter. HCV-229E RNA could be detected in some specimens and also the limiting number of targets detectable by this RT-PCR method was estimated at 60,000.

Peripheral blood lymphocytes from 13 healthy adults were analyzed for the presence of nucleic acid from both viruses and

three specimens of the 13 were positive in two consecutive tests for HCV-229E RNA. While this is a small number of specimens, this result is very promising in terms of future characterization of the tropism of HCV-229E. Failure to detect HCV-OC43 RNA indicates that these cells are not susceptible to infection by this virus, however, as in the case of HCV-229E, a larger number of individuals should be tested in order to confirm these observations. A study of CNS tissue specimens from multiple sclerosis patients and controls was also conducted. Four of 21 specimens from 11 MS patients were positive when tested for HCV-229E RNA and were confirmed positive in subsequent tests. None of 11 specimens from the control brains tested positive nor did any of the CNS tissues show evidence for the presence of HCV-OC43 RNA. Our results in CNS tissues emphasize the importance of continuing studies on the possible implication of HCV-229E in neurological diseases.

INTRODUCTION

Human respiratory coronaviruses, represented by strains 229E and OC43, have mainly been associated in the past with the common cold. They are estimated to be the cause of over 25% of colds (M^{c}Intosh *et al.*, 1974). They have also been associated with pneumonia, myocarditis and meningitis (Riski and Hovi, 1980). The observation of coronavirus-like particles in a brain section from a multiple sclerosis patient by Tanaka *et al.* (1976) has added human coronaviruses to the long list of viruses suspected of being involved in the etiology of multiple sclerosis. The existence of a murine model of demyelinating disease induced by murine hepatitis virus, also a coronavirus, suggested that human coronaviruses might produce a similar pathology in man in the form of multiple sclerosis. In 1980, Burks *et al.* isolated one coronavirus strain in each of two cultures of CNS specimens from MS patients, but these isolates were later shown to be more closely related to murine viruses than to either of the two human strains (Weiss, 1983). Nucleic acid hybridization was employed to probe for HCV-OC43 in four MS brains yielding negative results (Sorensen *et al.*, 1986). PCR technology now makes it possible to detect the very small amounts of viral nucleic acid which could be present in a disease of slow evolution such as MS in which the effects of a persistent infection of the CNS by a virus is suspected. In the present study, CNS tissues from MS patients and controls were analyzed by reverse transcription-polymerase chain reaction for

the presence of human coronaviral RNA from both strains of respiratory virus, HCV-229E and HCV-OC43. In addition, peripheral blood lymphocytes from healthy adults were tested for the presence of nucleic acid of these two viruses as a preliminary study of the feasibility of testing these tissues from MS patients.

LITERATURE REVIEW

1. *Coronaviridae*: Historical background

Of the human respiratory coronaviruses, HCV-229E and HCV-OC43 are characterized and partially sequenced to date. Along with human enteric coronaviruses (HECV), they belong to the family *Coronaviridae* which includes viruses causing disease in a wide variety of birds and mammals. Coronaviruses were first associated with avian infectious bronchitis and identified as the causative agent by Beaudette and Hudson in 1937. They observed characteristic particles by electron microscopy. Subsequently, coronaviruses infecting other species were identified: transmissible gastroenteritis virus causing diarrhoea in piglets (Doyle and Hutchins, 1946) and murine hepatitis virus by Cheever *et al.* (1949). The introduction of organ cultures (OC) of human embryonic trachea for the isolation of human cold viruses brought the discovery of coronaviruses infecting humans, the B814 strain isolated from a schoolboy suffering from a cold by Tyrrell and Bynoe in 1965. Other isolates of human coronaviruses causing infections of the upper respiratory tract were described by Hamre and Procknow, (1966), strain 229E, and by M^{c}Intosh *et al.* (1967), who described the OC strains related to OC43.

TABLE 1
CORONAVIRUSES: TYPES, HOSTS AND INFECTIONS

Virus	Host	Infection
antigenic group 1		
Human coronavirus 229E (HCV 229E)	humans	respiratory neurological?
Transmissible gastroenteritis virus (TGEV)	pigs	respiratory enteric
Porcine respiratory coronavirus (PRCV)	pigs	respiratory
Canine coronavirus (CCV)	dogs	enteric
Feline enteric coronavirus (FEPV)	cats	enteric
Feline infectious peritonitis virus (FIPV)	cats	respiratory, liver enteric, neurological, other*
antigenic group 2		
Human coronavirus OC43 (HCV-OC43)	humans	respiratory neurological?
Murine hepatitis virus (MHV)	mice	respiratory, liver enteric, neurological
Hemagglutinating encephalomyelitis virus (HEV)	pigs	respiratory, enteric neurological
Bovine coronavirus (BCV)	cows	enteric
Rabbit coronavirus (RbCV)	rabbits	enteric, other*
Turkey coronavirus (TCV)	turkey	respiratory enteric
Human enteric coronavirus (HECV)	humans	enteric?
antigenic group 3		
Avian infectious bronchitis virus (IBV)	chickens	respiratory, other*

*Other infections: infectious peritonitis, nephritis, pancreatitis, runting, parotitis and adenitis.

1.1 Taxonomy

The International Committee on the Taxonomy of Viruses recognized the family *Coronaviridae* with one genus, Coronavirus (Tyrrell *et al.*, 1975). Fourteen species have been identified to date (Table 1), the most recent addition being porcine respiratory coronavirus or PRCV (Cox *et al.*, 1990). While the original classification was based on the distinctive morphology of coronaviruses in terms of their size of about 100nm in diameter, pleomorphism, ether lability and the 20 nm projections on the virion surface as visualized under the electron microscope, subsequent studies of their structure and replication strategy have borne out the validity of their grouping within the family *Coronaviridae*. They are divided into three antigenic groups based on cross-reactivity of the different species. Sequence analysis has shown that, generally speaking, species that are closely related antigenically also exhibit a high degree of nucleotide identity (Verbeek *et al.*, 1991). However, a new group of viruses, the toroviruses, has been described, with Berne (BEV) as the prototype strain. These viruses exhibit strong similarities to *Coronaviridae* in terms of the organization of the genome and its expression (Snijder *et al.*, 1991) and appear to be ancestrally related (Snijder *et al.*, 1990). Their unique nucleocapsid morphology, the possession of unglycosylated membrane proteins and stability to

deoxycholate had originally justified their classification in a separate family (Horzinek *et al.*, 1987), but a recent review by the International Committee on the Taxonomy of Viruses (ICTV) has placed the separate genera of coronavirus and torovirus in the family *Coronaviridae* (David Cavanagh, personal communication).

Arteriviruses were previously classified as members of *Togaviridae*, but have recently been shown to produce a 3' coterminal nested set of mRNAs (Fig. 2), much like the *Coronaviridae*, suggesting that they also be reclassified in a manner which would relate them to this family of viruses (den Boon *et al.*, 1991). Indeed, arteriviruses were recently removed from the *Togaviridae* family and are awaiting inclusion into another family, most likely the *Coronaviridae* (David Cavanagh, personal communication).

2. Structure

Coronavirions vary from 100-150 nm in diameter, while in thin sections of infected cells, they appear as small as 85 nm. Club-shaped projections 20 nm in length, termed the peplomers, are visible on the outer surface (Fig. 1). They possess a phospholipid bilayer membrane and a coiled nucleic acid core. Coronaviruses are positive stranded RNA viruses, and typically of such viruses, the virion does not contain an RNA-dependent RNA-polymerase. Genomic RNA is a single strand 27 to 31

kilobases in length, making it the largest genome among RNA viruses studied to date (Lee *et al.*, 1991). In virions, genomic RNA is associated with several molecules of a basic phosphoprotein of 50 to 60 kDa, known as the nucleocapsid protein or N. Together N and genomic RNA form a long, flexible nucleocapsid with helical symmetry (Spaan *et al.*, 1988).

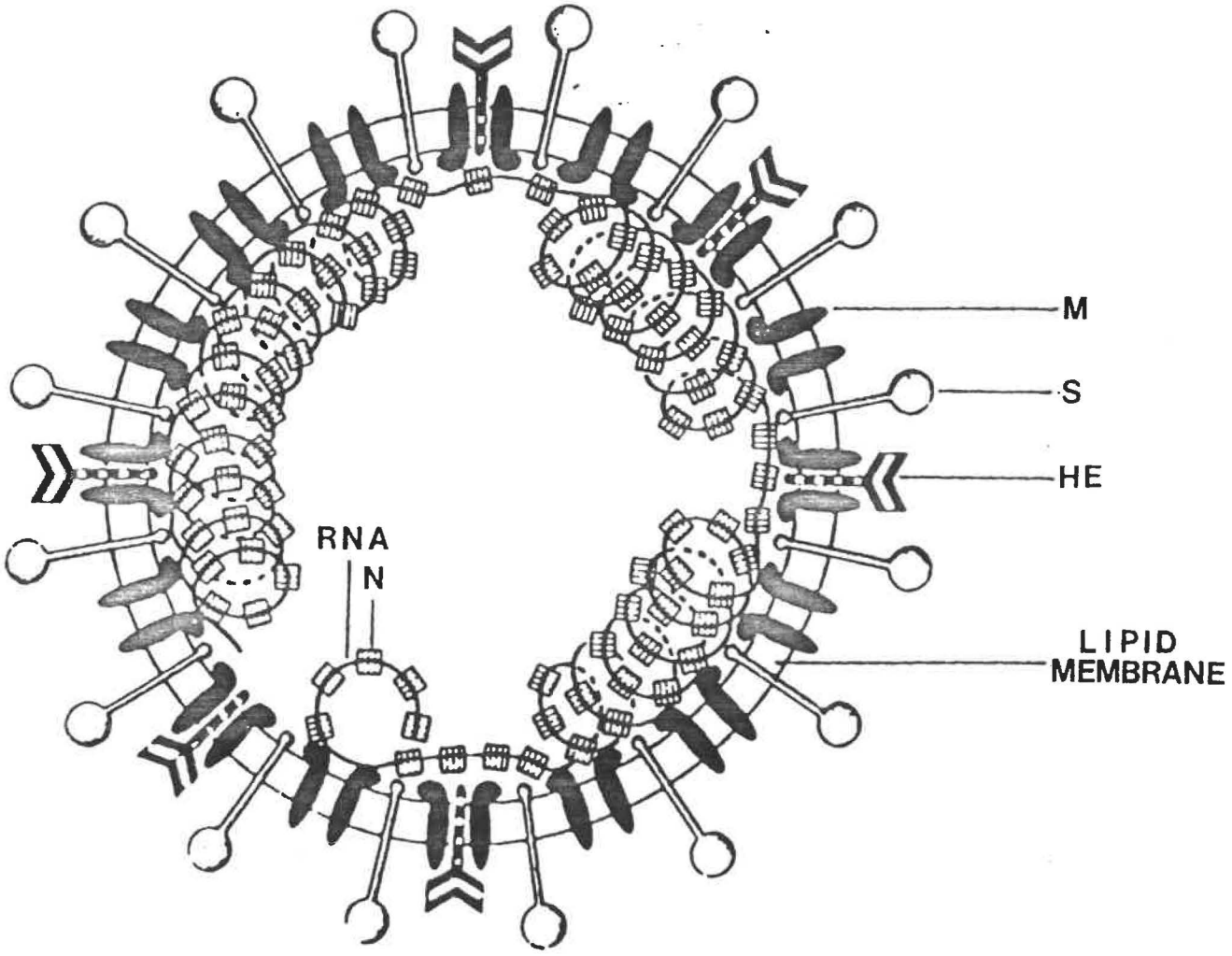
2.1 Structural proteins

All coronaviral species characterized to date contain three or four structural proteins, N as just described, M or membrane protein (formerly E1), S for surface protein (formerly E2) and some species, particularly OC43, also possess an HE or hemagglutinin-esterase protein (formerly E3) (Cavanagh *et al.*, 1990). A variety of functions have been ascribed to the four structural proteins.

2.1.1 Nucleocapsid protein (N)

As mentioned, N is associated with genomic RNA to form the nucleocapsid. In MHV, a specific interaction between nucleotides 56-65 of the leader sequence (see Replication, below) appears to be responsible for this association (Stohlman *et al.*, 1988). Analysis of the predicted amino acid sequences for N of five different MHV strains indicates that three regions that are highly conserved among the five strains may correspond to functional domains (Parker and Masters, 1990).

Figure 1: General structure of coronaviruses modified from Holmes, 1990.

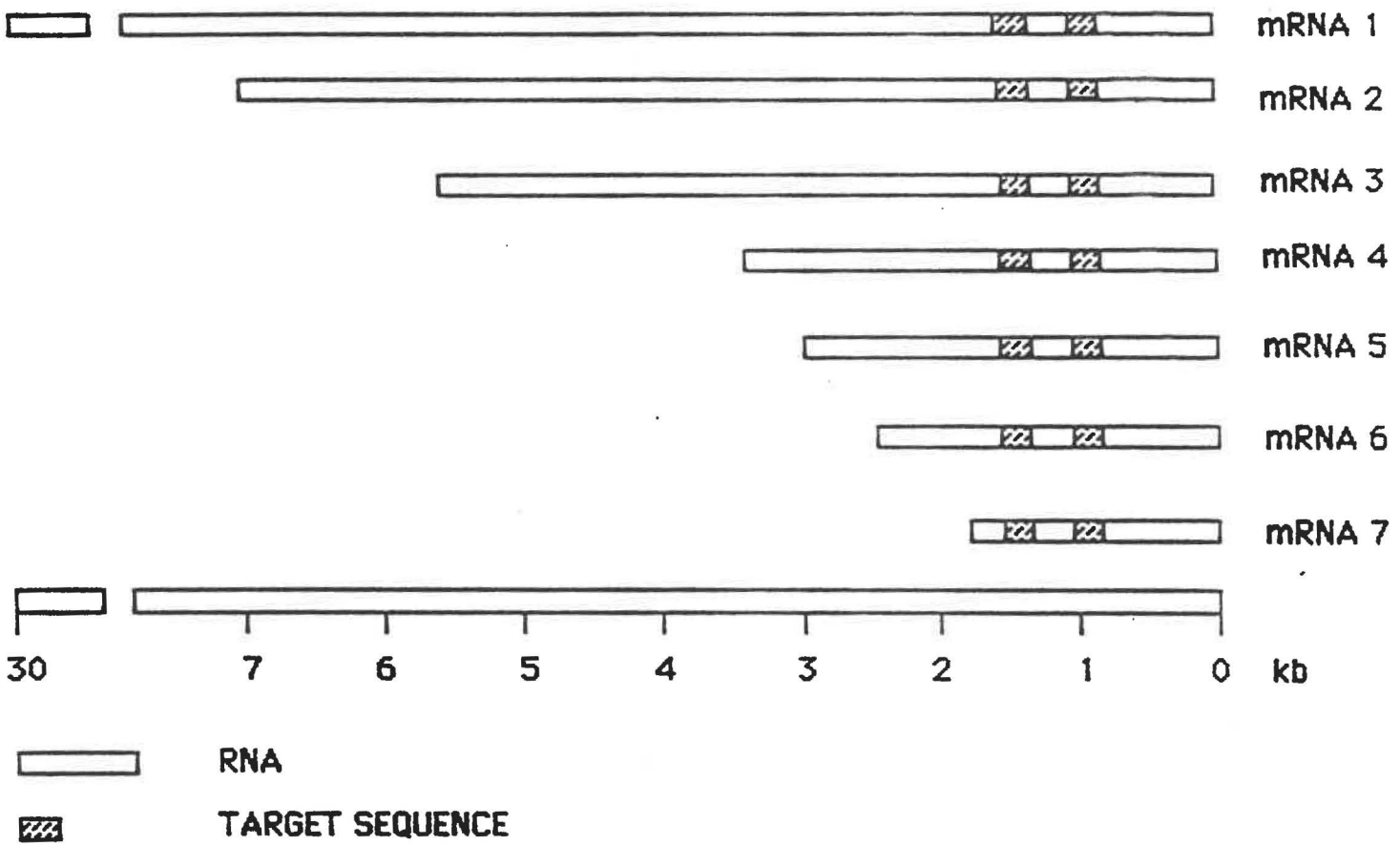

M
S
HE
RNA
N
LIPID
MEMBRANE

Two regions are highly basic while the third on the carboxy-terminal end is acidic. Numerous serine residues are present which are potential phosphorylation sites. The accumulation of this phosphoprotein in cellular infections may play a role in the switch from transcription to replication (Stohlman and Lai, 1979). Adoptive transfer of CD4+ T cells directed at epitopes on N protects rats against acute encephalitis when challenged intracerebrally with lethal doses of murine encephalitis virus (Körner *et al.*, 1991) indicating that N contains immunodominant epitopes.

2.1.2 Membrane protein

The deduced amino acid sequence of the M glycoproteins of IBV (Boursnell *et al.*, 1984), TGEV (Laude *et al.*, 1987), HCV-229E (Jouvenne *et al.*, 1990) and HCV-OC43 (Mounir and Talbot, 1992) indicate that it is a highly hydrophobic polypeptide with the potential to penetrate the lipid bilayer three times. Its *in vitro* binding to nucleoprotein (Sturman *et al.*, 1980) suggests that it is involved in encapsidation as well as in the induction of antibodies which neutralize viral infection in the presence of complement (Collins, 1982; Fleming *et al.*, 1989). The M glycoprotein is synthesized in the rough endoplasmic reticulum and is then transported to the Golgi apparatus where it is glycosylated. Glycosylation of M is *O*-linked in MHV, BCV and OC43 (Holmes, *et al.* 1981; Deregt *et al.*, 1984; Mounir and

Figure 2: 3' coterminal nested set of mRNAs expressed by HCV-229E and location of target sequences corresponding to primers #1 and #2.


mRNA 1
mRNA 2
mRNA 3
mRNA 4
mRNA 5
mRNA 6
mRNA 7
30
7
6
5
4
3
2
1
0
kb
RNA
TARGET SEQUENCE

Talbot, 1992) and *N*-linked in IBV and TGEV (Laude *et al.*, 1987; Stern and Sefton, 1982). One potential *N*-glycosylation site (Asn-5) and three *O*-glycosylation sites (Ser-2, The-7, Thr-12) are found near the presumed externally exposed N-terminus of M of HCV-229E based on the predicted amino acid sequence (Jouvenne *et al.*, 1990), but glycosylation is sensitive to tunicamycin, indicating that N-glycosylation predominates (Arpin, 1990). M appears to play a role in virus budding, which takes place in the Golgi apparatus where M accumulates.
Induction of α-interferon by TGEV has been attributed to a restricted region of the M glycoprotein at the presumed externally exposed N-terminus. Each of two complement-mediated neutralization escape mutants which did not induce α-interferon possessed a point mutation in this region, resulting in a single amino acid change, Thr-17 to Ile in one case and Ser-19 to Pro in the other (Laude *et al.*, 1992).

2.1.3 Surface protein (S)

The peplomer glycoprotein of 180 to 200 kDa is responsible for the crown-like appearance of the virions. In TGEV, three molecules of S are noncovalently linked to form the spike (Delmas and Laude, 1990), which is probably the case for other coronaviruses. It is the carboxy terminal half of S which is anchored in the lipid membrane. A number of critical functions are attributed to this glycoprotein: attachment to a virus

receptor on the cell, the induction of cell fusion and the possession of epitopes responsible for the induction of neutralizing antibodies (Collins *et al.*, 1982; Daniel *et al.*, 1990). In some coronaviruses such as MHV, S is cleaved into two subunits termed S1 and S2 for the N- and C-terminal ends, respectively, by host cell proteases during virus maturation. In MHV, this cleavage is required for inducing fusion activity (Sturman *et al.*, 1985). S is responsible for binding to a member of the carcinoembryonic antigen family (Williams *et al.*, 1991), a 110 kDa cellular receptor for MHV (Boyle *et al.*, 1987) and is thus essential for viral infectivity. Studies with monoclonal antibody escape mutants have allowed the identification of epitopes involved in the neuropathogenicity of MHV-JHM. A single nucleotide mutation at nucleotide 3340, in the S2 subunit appears to be capable of eliminating the encephalitic potential of JHM DL (Wang *et al.*, 1992). Previous reports of vaccination against encephalitis with a decapeptide coded for by nucleotides 2979-3006 (Talbot *et al.*, 1988) suggest that this region of S is indeed involved in producing encephalitis. A 102-nucleotide deletion in S1 eliminated the demyelinating potential of the escape mutant (Wang *et al.*, 1992), thus pinpointing the regions of the S glycoprotein responsible for the neuropathogenic properties of MHV-JHM. Rats with MHV-JHM-induced subacute demyelinating encephalomyelitis produce CD4+

T-cells specific for the S protein which protected suckling rats from a lethal dose of MHV-JHM (Körner *et al.*, 1991).

2.1.4 Hemagglutinin-esterase

This glycoprotein has been found in BCV, HCV-OC43, TCV and in some strains of MHV. It binds to 9-*O*-acetylated neuraminic acid residues on the cell membranes, particularly on erythrocytes. It also possesses acetylesterase activity which causes the elution of hemagglutinated virus particles. Homology between the nucleotide sequence of the protein of coronaviruses including HCV-OC43 (Zhang *et al.*, 1992) and that of influenza C hemagglutinin is often cited as an example of the ability of coronaviruses to recombine during replication (Luytjes *et al.*, 1988). The HE glycoprotein is responsible for hemadsorption and hemagglutination of erythrocytes. It remains to be determined whether this molecule may also play a role in attachment to the cells upon infection.

3. Replication

Most knowledge of coronaviral replication comes from studies of murine hepatitis virus strains and may be extrapolated to other members of *Coronaviridae*, in this case to HCV-229E and HCV-OC43. As previously mentioned, viruses are presumed to attach to some cells via the S glycoprotein. However, viruses with an HE glycoprotein may also attach

through it, although this has not been shown. It is not known whether penetration of the host cell is achieved by endocytosis or by fusion of the lipid bilayer of the virion with the cell membrane mediated by the fusion properties of the S glycoprotein. Coronavirus infectivity is attenuated by the lysosomotropic drug ammonium chloride (Mizzen *et al.*, 1985) delaying viral mRNA production and protein synthesis five hours. This suggests that fusion with the endosomal membrane likely occurs in the absence of this compound, thus favoring endocytosis as the mechanism of host cell penetration. However, recent evidence suggests fusion at the plasma membrane also occurs (Kooi *et al.*, 1991).

For coronaviruses, the first stage of replication has not yet been elucidated. Indirect evidence suggests that the genomic RNA, which is both capped and polyadenylated, is translated by the existing cellular apparatus. The product of translation would be the polypeptide(s) coded by the first gene on the 5' end of the genome, which is/are believed to possess the RNA-dependent RNA polymerase activity required for genome replication, i.e. the synthesis of strands of negative polarity which in turn serve as the template for the synthesis of the positive strands of genomic and messenger RNAs. While this gene has the capacity to code for a 700 kDa polypeptide, for MHV, a 250 kDa precursor is detectable which is then cleaved into two

250 kDa precursor is detectable which is then cleaved into two polypeptides of 220 kDa and 28 kDa (Denison and Perlman, 1986).

Five to eight species of subgenomic mRNAs are transcribed. They form a nested set of RNAs which share their 3' ends but which extend for increasing lengths toward the 5' end of genomic RNA such that each RNA contains an ORF at its 5' end which is not present on the next smaller mRNA species (see Fig. 2). The *Coronaviridae* Study Group for the Nomenclature of the Structural Proteins, mRNAs and Genes of Coronaviruses has recommended that genomic RNA be named RNA 1 and that the successively smaller RNAs be named in ascending order (Cavanagh *et al.*, 1990). It has been shown for MHV that RNAs of similar size but of negative polarity are also transcribed during infection but in vastly smaller quantities than their positively-stranded counterparts (Sawicki and Sawicki, 1990). They likely serve as the template for the synthesis of mRNAs, based on the fact that no free negative stranded RNA is detectable in infected cells, it is only found in the form of double-stranded RNA. The same is probably true for other species of coronaviruses, although it has only been confirmed for TGEV to date (Sethna *et al.*, 1989).

3.1 Transcription strategy

Transcription of RNA in coronavirus replication as well as mRNA synthesis appears to be leader primed. This model was proposed by Lai (1986) and is based on a number of experimental observations. All mRNAs of MHV and IBV including the genome possess an identical sequence, 60-70 nucleotides in length at the 5' end (Brown *et al.*, 1984; Lai *et al.*, 1984). Eight to ten nucleotides in these sequences (termed leader RNA) are homologous with the consensus intergenic sequence UCUAAAC, which is present in the region preceding each gene on mRNA. Partly overlapping the consensus sequence are several pentanucleotide repeats UAAUC. This sequence present close to the 3' end of leader RNA, is hypothesized to be responsible for the binding of the leader to the negative stranded template by complementary base-pairing. A mismatch is often present in the consensus sequence which could serve as a site for the cleavage of leader RNA leaving a free 3' end on the bound leader to serve as a primer for RNA polymerase. If indeed mRNAs are synthesized on a template of the same size, then the consensus sequence may also serve as a signal to stop transcription of the genomic template during the synthesis of the negative strands.

There is substantial evidence that a free leader RNA primes RNA transcription. We have already seen that the same

sequence of 60 to 70 nucleotides is present at the 5' end of every mRNA species. In addition to mRNA, a 50-90 nucleotide RNA is detectable in MHV infected cells (Baric *et al.*, 1985). The same group has shown that a temperature-sensitive mutant only synthesizes this small RNA species at the non-permissive temperature. Mixed infections with different MHV strains results in 40 to 50% hybrid mRNAs which possess the leader sequence of the coinfecting strain at the 5' end, suggesting that leader sequences are incorporated at random, independently of the source of template RNA (Makino *et al.*, 1986). Finally, an *in vitro* transcription system for MHV incorporating synthetic leader RNAs into viral mRNA during its synthesis has confirmed the existence of the leader-primed transcription strategy of coronaviruses and has further clarified the role of the pentanucleotide repeats, as necessary for binding to template (Baker and Lai, 1990). Mismatches between leader and template were corrected in this system, indicating an enzymatic activity related to correction of viral RNA transcription associated with the virus.

3.2 Morphogenesis

The synthesis of S and HE appears to be similar to that of other viral glycoproteins. They are inserted through the membrane of the rough endoplasmic reticulum (RER) and glycosylated cotranslationally (Klenk and Rott, 1980). S is

then either incorporated into virions or transported to the plasma membrane, making the cell susceptible to lysis and permitting cell fusion, which is mediated by this glycoprotein (Sturman and Holmes, 1984). Unlike many enveloped viruses, coronaviruses appear to bud intracellularly at the membranes between the RER and the Golgi apparatus. It appears that N is synthesized on ribosomes in the cytoplasm of the host cell where it interacts with genomic RNA to form nucleocapsids. They then interact with RER or Golgi membranes, probably by binding to M. M is synthesized on membrane-bound polysomes and the first membrane-spanning domain is believed responsible for its targetting to the cis Golgi (Machamer *et al.*, 1990). Glycosylation of M occurs in the Golgi apparatus well after protein synthesis has taken place (Niemann *et al.*, 1982). Virions bud intracellularly in the absence of glycosylated S and HE, and in fact S and HE may not be necessary for the formation of virions. Intracellular formation of virions may be important for allowing persistence in an immune host. Virions exit by lysis of dying cells, but they also appear to utilize the cellular secretory apparatus (Sturman and Holmes, 1983). Virions are transported to the periphery of the cell in smooth-walled vesicles where the vesicles likely fuse with the plasma membrane to release the mature virions.

4. Tropism and pathogenesis

Coronaviruses exhibit highly specific tissue tropism and tend to infect one host species or a few closely related species (see Table 1). Many coronaviruses are strictly limited to infections of epithelial cells of the respiratory or intestinal tract or both while others such as MHV and FIPV infect the respiratory and intestinal tracts, the brain, the liver, and, in the case of MHV, macrophages and lymphocytes as well. The cellular receptor for MHV on macrophages, hepatocytes and cells of the intestinal epithelium has been identified and is a member of the carcinoembryonic antigen family of proteins (Williams *et al.*, 1991). Since it is not present on neurons or oligodendrocytes in the CNS of mice, it is postulated that one or more additional receptors are employed by MHV to infect these cells.

4.1 Neurotropism

The neurologic tropism of MHV is the focus of a great deal of interest as chronic infection with MHV serves as an animal model for demyelinating diseases such as multiple sclerosis. The outcome of inoculation of mice with neurotropic strains of MHV depends on the age and strain of mouse, the strain of virus, the route of inoculation and dose of inoculum. Many models of chronic demyelination are presently in existence,

particularly the use of MHV-A59 inoculated intracerebrally to produce biphasic disease in C57BL/6 mice, MHV-JHM intranasal inoculation of C57BL/6 mice suckling from immunized dams (Perlman *et al.*, 1987), MHV-JHM intracerebrally inoculated in BALB/c mice (Knobler *et al.* 1982) and MHV-A59 in weanling C3H mice (Woyciechowska *et al.*, 1984). Temperature-sensitive mutants of both MHV-A59 and MHV-JHM have been generated to selectively produce demyelination in order to elucidate the mechanisms involved in this virally-induced process (Haspel *et al.*, 1978; Koolen *et al.*, 1987). Monoclonal antibodies to different epitopes on the S glycoprotein have been successfully employed to select for genetic variants with differing neuropathogenic properties (Dalziel *et al.*, 1986; Fleming *et al.*, 1986), one causing acute encephalitis like the parent strain of MHV-JHM and another causing paralysis with histological evidence of extensive demyelination.

4.2 Pathogenesis of MHV-induced demyelination

Several mechanisms for virally-induced demyelination have been postulated: direct viral injury to oligodendrocytes, the myelin producing cells, and immune-mediated mechanisms (Johnson, 1985). These include the bystander theory whereby myelin or oligodendrocytes in the vicinity of virally infected cells are destroyed by cathepsins or phospholipases released by macrophages during an immune response mounted against viral

antigens, immunologic injury to oligodendrocytes infected with virus (Rodriguez, 1989) and autoimmune events related either to the release of myelin components during the course of viral infection, the presentation of self-antigens incorporated in virions during replication (Anomasiri *et al.*, 1990) or molecular mimicry in which the sharing of epitopes between virus antigens and myelin components elicits an immune response against self-antigens.

Experimental evidence in support of both direct viral injury to oligodendrocytes and of the stimulation of autoimmune events has been documented for MHV-JHM infections and to a lesser extent for MHV-A59. The classic scenario of MHV infection is an acute encephalitis typically exhibiting infection and destruction of neurons resulting in death in the majority of affected mice. Survivors exhibit persistent infection of oligodendrocytes but not neurons accompanied with demyelination of a chronic or subacute type in CNS white matter. Fatal encephalitis can be modulated when suckling mice of immunized dams are inoculated resulting in chronic demyelination (Perlman *et al.*, 1987). Likewise, intracerebral inoculation of C3H mice, a strain resistant to fatal encephalitis, results in acute and subacute demyelination with subtle neurologic symptoms (Woyciechowska *et al.*, 1984). Administration of monoclonal antibody, directed to a

neutralizing epitope on the S protein, also obviates fatal encephalitis and results in demyelination upon challenge with wild type strain of MHV JHM (Buchmeier *et al.*, 1984). It has long been hypothesized that the demyelination observed in MHV infections is due either to direct injury by the virus to oligodendrocytes, or to an autoimmune response triggered by viral infection through the mechanisms described above. After the inoculation of MHV-JHM, antigen is detected in oligodendrocytes 365 days later (Knobler *et al.*, 1981). It has also been demonstrated that MHVs are capable of infecting oligodendrocytes *in vitro* in a persistent fashion (Lavi *et al.*, 1987). On the other hand, there is evidence in favor of autoimmune stimulation by MHV as the principal mechanism producing demyelination. When intracerebral inoculation of MHV-A59 is employed, viral nucleic acid, but not viral antigen, is detectable in oligodendrocytes of the affected CNS white matter as late as ten months post-inoculation (Lavi *et al.*, 1984). Watanabe *et al.* demonstrated in 1983 that subacute demyelinating encephalomyelitis following intracerebral inoculation of MHV-JHM in Lewis rats could be produced in healthy rats after adoptive transfer experiments in which lymphocytes of diseased rats were stimulated with myelin basic protein *in vitro* prior to transfer. When intranasal inoculation of the JHM strain is performed on suckling mice of immunized dams, foci of demyelination occur in regions of the brain where

no viral nucleic acid is detectable by *in situ* hybridization (Perlman *et al.*, 1988). Yet another study strongly indicates that immunopathological mechanisms directed against viral antigens are principally responsible for demyelination in JHM infections (Wang *et al.*, 1990). After irradiation 6 days post-infection, infected mice had even higher titers of virus in the brain than unirradiated control mice, but showed marked reductions in paralysis and demyelination. Furthermore, adoptive transfer of T lymphocytes from infected to infected, irradiated mice but not to naive, irradiated mice produced demyelination suggesting that a product of viral infection such as antigen is necessary as a target in demyelination. The same experiments were repeated in BALB/c mice using another viral variant demonstrating that the phenomenon is not dependent on the mouse strain or the virus variant employed. However it is clear that there is potential for a number of mechanisms to be at work simultaneously or possibly in a cascading sequence in MHV-induced demyelination. Kyuwa *et al.* (1991) have recently reported a three- to tenfold increase in the frequency of autoreactive IL-2-secreting T cell clones following murine hepatitis infection produced by intraperitoneal inoculation. If confirmed, this may well constitute an additional mechanism by which an autoimmune reaction could be initiated by viral infection. An unexpected result in the study by Wang *et al.* (1990) may be related to this phenomenon. The authors found

that when demyelination was elicited once again in irradiated mice by adoptive transfer of T lymphocytes from infected mice, control T cells from naive mice also produced a similar degree of demyelination. Subtle interactions between virus and the immune system in these models seem to determine the final outcome of infection.

4.3 Tropism of Human Coronavirus

Human coronaviruses OC43 and 229E were first associated with the common cold and are known to replicate in epithelial cells of the respiratory tract. Whether they are capable of infecting other human cell types is unclear, although HCV-OC43 can be cultured in suckling mouse brain and in primary mouse embryo brain cultures in which fibroblasts and astrocytes were infected (Pearson and Mims, 1985). Adaptation of strain OC43 for growth in tumor cell lines derived from human glioblastoma and rhabdomyosarcoma and the establishment of persistent infection in them has also been reported (Collins and Sorensen, 1986). The possibility that human coronaviruses may be neurotropic has come under consideration mainly due to studies on multiple sclerosis.

In 1976, Tanaka *et al.* observed coronavirus-like particles in the perivascular cuffing of a CNS plaque from a multiple sclerosis patient. The particles were observed in the cisterns

of the RER of large mononuclear cells of the cuff. Their diameter ranged from 55 to 65 nm, corresponding to the size of coronaviruses excluding the 20 nm spikes which were not visible in the electron micrograph. Despite their location in the RER, no particles were observed budding from the RER membranes as would be expected in a cellular infection by coronavirus. Similar particles were not observed in eleven other brains of multiple sclerosis patients. Whether these particles were transported to the brain from another site, the cell was itself infected with coronavirus or the particles had been taken up by the monocyte at that site is impossible to tell.

In our laboratory, *in vitro* infection of human neuroblastoma cell lines IMR-32 and SK-N-SH with HCV-229E detected by immunofluorescence indicates that neurons may bear receptors for and be susceptible to infection by this strain of virus. Another cell line, MO3-13, which was established by Dr. Neil Cashman from a fusion of human oligodendrocytes and the rhabdomyosarcoma cell TE-671 (RD), also supports HCV-229E replication. Evidence is weaker in the latter case because the parent TE-671 cells alone could also be infected by this virus, although to a lesser degree than the fusion-derived MO3-13 cell line (Ékandé *et al.*, unpublished results).

Attempts to isolate coronavirus in cell cultures from MS white matter at autopsy by Burks *et al.* in 1980 led to the

report of viral isolates from two different patients. It was later shown that the two isolates shared greater nucleotide homology with murine hepatitis virus than with either human coronavirus (Weiss *et al.*, 1983). While this may suggest that a coronavirus more closely related to MHV than either OC43 or 229E is involved in the pathogenesis of MS it could also indicate that a laboratory contamination of the mouse cell line used for virus culture with MHV was responsible for these results. One of the isolates, termed SD, has been shown to produce demyelination in primates after intracerebral inoculation (Murray *et al.*, 1992b). Whether this is a human virus very closely related to murine coronaviruses or whether the same virus infects both mice and humans remains to be determined. Thus, the question as to whether coronaviruses may be implicated in MS remains open as does the hypothesis of a viral etiology of multiple sclerosis.

5. Multiple sclerosis: an autoimmune disease

The characteristic lesion in multiple sclerosis, the plaque or zone of demyelination is often examined for the first clues as to the etiology of the disease. A variety of immune effector cells are observed such as macrophages containing myelin debris at the center of demyelination and T lymphocytes in perivascular cuffings at the edges of active plaques, which may be predominantly CD4+ or CD8+ and which bear IL-2 receptors

(Sobel, 1989). The expression of Class I and II MHC antigens on astrocytes at plaque borders differs greatly from normal white matter in which Class II antigens are only detected on endothelial cells, if at all (Traugott, 1987). Thus, there is an ongoing immune response which is likely responsible for the demyelination; however it is not clear what initiates demyelination and what aspects of the immune response are epiphenomena.

A broad spectrum of immune reactions has been documented in MS patients which are of probable importance to its pathogenesis but not necessarily to its etiology. The presence of oligoclonal IgG bands in the CSF of multiple sclerosis patients is not specific to MS. High levels of immunoglobulin secreting cells to myelin basic protein were found in the CSF of 57% of MS patients but also in the CSF of patients with other neurological diseases (Link *et al.*, 1990). Elevated levels of IL-6 are detected in the plasma but not in the CSF of 93% of MS patients (Frei *et al.*, 1991). Cells secreting anti-myelin associated glycoprotein were found in the CSF of 48% of MS patients but not in patients of other inflammatory neurological diseases (Baig *et al.*, 1991). Recently, T cells reactive to myelin oligodendrocyte glycoprotein were observed in the CSF of 12 of 14 MS patients and B cells with the same reactivity in 8 of 10 patients (Sun *et al.*, 1991) while such

cells were found in two of 18 controls, and were less frequent. Reactivity to mitogen is not enhanced in MS. However, enhanced stimulation of CD45RA+ T cells, termed "naive" T cells, by activated CD8+ cells from MS patients has been observed (Freedman *et al.*, 1991). Unlike other antibody to MBP and PLP, anti-MOG mAb produces demyelination both *in vivo* and in aggregating brain cell cultures (Kerlero de Rosbo *et al.*, 1990), suggesting a possible role in demyelination for antibodies of this specificity.

Much attention has been paid to MBP reactive T-cells in MS patients. While the frequency of such cells appears to be higher in MS patients both in peripheral blood (Allegretta *et al.*, 1990) and in the CSF (Olsson *et al.*, 1990) as compared with aseptic meningitis or tension headache patients, such reactivities are present in normal individuals.

5.1 Evidence for genetic factors influencing susceptibility to multiple sclerosis

The prominence of the autoimmune response and aberrant immune responsiveness in MS suggest a genetic predisposition for multiple sclerosis determined by the interplay of MHC and other susceptibility and resistance genes (Ebers *et al.*, 1986). A higher concordance for the rate of multiple sclerosis in monozygotic than dizygotic twins (26% vs. 2.3%) indicates that genetic factors play a role (Ebers *et al.*, 1986). This

conclusion is often criticized because the concordance for this rate in monozygotic twins is less than 50%. However, great variability in the age of onset and the random manner in which lesions progress in different areas of the brain without necessarily being expressed symptomatically may explain at least some of the discordance.

Establishing firm associations between candidate susceptibility genes and risk for the disease has proved much more complex. In northern European and north American Caucasian populations a loose association with HLA-DR2/Dw2 antigens has been found with a relative risk of between two to four (Compston, 1986). In other populations, different haplotypes have been associated with the disease such as HLA-DR4 in Jordanian Arabs and HLA-DRw6 in Japanese and Mexican populations (Oksenberg and Steinman, 1990). The use of restriction fragment length polymorphism (RFLP) analysis of MHC class II alleles in MS and controls (Vartdal, 1989; Haegert, 1990) is making it possible to characterize genetic similarities encountered in populations of MS patients at the molecular level. However, the presence of the same RFLPs associated with MS at a lower proportion in the general population suggests that MHC Class II genes alone do not determine susceptiblity to MS (Vartdal, 1989).

More recently, T-cell receptor Vα and Vß usage, first studied in T-cell clones to myelin basic protein in experimental allergic encephalomyelitis, has been studied in MS patients and a limited number Vα transcripts, 8,9,10,12 and 16 were amplified in MS brains (Oksenberg *et al.*, 1990). Wucherpfennig *et al.* (1990) found over 50% of ß chains from T-cell lines reactive to MBP (84-102) employed Vß17 while Ben-Nun *et al.* (1991) found the usage of a single, but different Vß chain predominated in each of the individuals tested. An attempt to localize the putative susceptibility gene in 38 unrelated MS patients indicated a locus mapping between Vß-8 and Vß-11 within the TCR ß complex (Charmley *et al.*, 1991).

It is hypothesized that in autoimmune disease the induction of a T-cell response to self-antigens such as myelin basic protein is determined by the trimolecular complex formed between the antigenic peptide in the context of MHC molecules and the T-cell receptor. Certain combinations of T-cell receptor and HLA antigens may determine susceptibility for a breakdown of the regulation of self-tolerance and the development of a specific immune response against a self-antigen (Bell *et al.*, 1990). There is doubt as to whether self-tolerance to any of the myelin antigens is generated since myelin is formed later in development, and may not be present in the thymus during induction of self-tolerance in the foetus.

Additionally, such interactions could also be of importance in autoimmune responses triggered by infection with a pathogen sharing homology with a self-antigen. Other genetic factors of potential importance are genes regulating the inducibility of MHC molecules on astrocytes or on endothelial cells in the CNS, given that the initiation of an immune response could be provoked by the presentation of antigen on these cells in the context of MHC assuming that self-reactive clones are present in the circulation. Massa *et al.* showed in 1987 that the inducibility of Ia antigen on rat astrocytes by murine coronavirus JHM is rat strain-dependent and that it correlates with susceptibility to demyelinating disease. Also, MHV-A59 has been shown to induce MHC Class I but not Class II antigens on both oligodendrocytes and astrocytes of BALB/c and C57BL/6 mice (Suzumura *et al.*, 1988). Interestingly, a transgenic mouse model for CNS demyelination by a non-inflammatory mechanism has been reported recently. The selective expression of Class I MHC antigen on oligodendrocytes was found to result in widespread demyelination in the CNS with higher or normal expression of MBP mRNA (Yoshioka *et al.*, 1991). Reduced suppressor activity during active disease in MS (Antel *et al.*, 1978) could also be genetically determined and favor the development of an autoimmune response.

Vertically transmitted endogenous retroviruses have recently come under examination as a possible cause of

autoimmune phenomena in multiple sclerosis since the discovery that the long terminal repeat of Mouse Mammary Tumor Virus encodes a protein with a self-superantigen activity in mice (Choi *et al.*, 1991). The expression of such a superantigen in the CNS, particularly by oligodendrocytes, could potentially trigger an on-going autoimmune reaction against myelin (Antel and Cashman, 1991).

5.2 Evidence for an environmental factor as etiologic agent of multiple sclerosis

Epidemiologic studies suggest that an as of yet unidentified environmental factor is required to initiate the disease process in multiple sclerosis. A number of observations point to an environmental agent acting in individuals years before the disease declares itself. Frequently, the generalization is made that the prevalence of multiple sclerosis describes a north-south gradient whereby the rate increases with degrees latitude north or south of the equator (Saint and Sadka, 1962; Johnson, 1985). While many exceptions and variations exist (Williams, 1991; Skegg, 1991), studies of migrants from high to low prevalence regions have established that migration before the age of 15 confers a risk for multiple sclerosis corresponding to the country of adoption (Dean and Kurtzke, 1971; Alter, 1966). Individuals migrating after the age of 15 maintain the same level of risk as existed

in their native country (Kurtzke, 1991). This would imply either that exposure to an environmental agent before the age of 15 initiates the disease process, or that exposure to an agent before the age of 15 confers protection against disease when exposed later in life (Kurtzke, 1971).

The occurence of cases of multiple sclerosis on the Faroe Islands for the first time in 1943 is a strong indication that indeed an environmental factor is necessary to initiate the disease process in susceptible individuals (Kurtzke and Hyllested, 1986). Presumably, this environmental agent would have been introduced in the islands a period of time prior to the occurence of the first cases of multiple sclerosis.

5.2.1 Multiple sclerosis: the viral hypothesis

The search for the putative causal agent of multiple sclerosis continues as no single agent has been consistently detected to date. Among infectious agents, viruses are prime candidates due to the documented evidence of slow, demyelinating viral infections of both animals (coronavirus, visna virus and Theiler's virus of mice) and humans (subacute sclerosing panencephalitis caused by measles virus, progressive multifocal leukoencephalopathy caused by JC virus, and more recently, HTLV-I in tropical spastic paraparesis/HTLV-I asssociated myelopathy TSP/HAM). Antibody to a number of

viruses including both HCV-229E and HCV-OC43 is detectable in sera as well as in the cerebrospinal fluid of multiple sclerosis patients, some individuals possessing antibody in the cerebrospinal fluid to up to 11 different viruses simultaneously (Salmi *et al.*, 1983), however this abnormal response to viral antigens is not specific to multiple sclerosis alone. In the case of antibody to measles at least, low affinity antibodies to the virus are synthesized in MS patients, which is not indicative of ongoing infection (Luxton and Thompson, 1990).

5.2.2 Multiple sclerosis: virus isolation

Many reports of virus isolation from multiple sclerosis tissue have appeared, but none have been confirmed in subsequent studies. Rabies virus (Margulis *et al.*, 1964), Herpes simplex virus (Gudnadottir *et al.*, 1964), parainfluenza virus 1 (ter Meulen *et al.*, 1972), measles virus (Field *et al.*, 1972), Simian virus 5 (Mitchell *et al.*, 1978), cytomegalovirus (Wrobleska *et al.*, 1979), coronavirus (Burks *et al.*, 1980) subacute myelo-optico-neuropathy-like virus (Melnick *et al.*, 1982) and tick-borne encephalitis virus (Vagabov *et al.*, 1982) have each been implicated in multiple sclerosis at different times.

There is a continued search for the implication of new human retroviruses in MS due to the neuropathic potential of known retroviruses. Isolation of retroviruses from mononuclear cells of the peripheral blood of MS patients has been attempted (Kam-Hansen *et al.*, 1989) and both cytopathic effects and reverse transcriptase (RT) activity were detected in cultures from two of 15 MS patients and none of 15 healthy controls. Long-term culture of mononuclear cells from 33 MS patients did not yield supernatants with RT activity (Höllsberg *et al.*, 1989). A different group, after isolating a retrovirus from the cerebrospinal fluid of one MS patient (Perron *et al.*, 1989), cultured monocytes from the peripheral blood of 18 MS patients and tested the supernatants for reverse transcriptase activity. Ten of 18 patients had RT activity above the control-based cut-off value in at least one culture (Perron *et al.*, 1991). Observation of cell pellets by electron microscopy revealed retrovirus-like particles in RT positive, but not in RT negative cultures.

5.2.3 Multiple sclerosis: hybridization to detect viral nucleic acid

Nucleic acid hybridization techniques have been used to test multiple sclerosis brain tissue for the presence of measles virus RNA, giving vastly different results in three studies. In 1981, Haase *et al.* detected two foci of measles

virus genomes in one of four multiple sclerosis brains by *in situ* hybridization. An attempt to detect measles virus RNA by both dot blot and *in situ* hybridization in 11 multiple sclerosis brains was unsucccessful, although the genome of this virus was easily detected in SSPE brains (Dowling *et al.*, 1986). Finally, Cosby *et al.* (1989) detected measles virus nucleic acid in two of eight cases of multiple sclerosis and in one case of disseminated cytomegalovirus infection by *in situ* hybridization. Six of 30 blocks and four of 36 blocks were positive in the two multiple sclerosis brains, particularly in periplaque tissue. Hybridization has also been applied in the detection of coronaviruses, notably the analysis of the brains of four MS patients for the presence of the genome of OC43 RNA by Sorensen *et al.* (1986), which produced negative results. R.S. Murray, of J.S. Burks' group, has reported the detection of coronaviral genomes in 11 of 21 brains of multiple sclerosis patients and in 2 of 21 control brains by *in situ* hybridization using a clone of their SD 1980 multiple sclerosis isolate as a probe. They obtained similar results using an MHV-A59 clone as a probe (Murray *et al.*, 1990). A more in-depth study of these tissues using probes for Herpes simplex virus, Theiler's murine encephalomyelitis virus and HCV-229E in addition to the SD and MHV-A59 probes gave the same results for the latter two viruses and were negative for the others (Murray *et al.*, 1992a).

5.2.4 Multiple sclerosis: Detection of viral nucleic acid using the polymerase chain reaction.

The advent of PCR technology has resulted in a new generation of studies of multiple sclerosis tissues for the presence of viral genomes. It is hypothesized that the slow progression of the disease and the long latency period may be due to a persistent, rather than a fulminant, viral infection and since PCR can potentially detect very low numbers of molecules, it may finally be possible to detect the multiple sclerosis virus, or its genome, where older methods of virus isolation and hybridization have fallen short of this goal (Brahic and Jewell, 1989).

The initial results with PCR in detecting human T lymphotrophic virus I (HTLV-I) provirus in peripheral blood lymphocytes of multiple sclerosis patients (Reddy *et al.*, 1989) was first challenged by Bangham *et al.* (1989) on two counts. Firstly, Reddy's group had found identical sequences in the amplified region of the *env* gene in all six patients with positive results. This contrasted with individual variations found in this region at the nucleotide level in isolates from different patients. Secondly, results from Bangham's laboratory in analyzing nine multiple sclerosis patients using *env*- and *pol*-specific primers, were all negative. Subsequent studies of the peripheral blood lymphocytes and CNS tissue of multiple

sclerosis patients by PCR for homologous HTLV-I sequences failed to find any (Richardson *et al.*, 1989; Oksenberg *et al.*, 1990; Fugger *et al.*, 1990; Jocher *et al.*, 1990; Nishimura *et al.*, 1990; Kaneko *et al.*, 1991; Prayoonwiwat *et al.*, 1991). The HTLV-MS Working Group was formed to analyze the evidence from such studies, and a large-scale, blind study was set up involving over 1005 patient specimens, 202 of which were from multiple sclerosis patients. The only confirmed positive specimen in this study was from a tropical spastic paraparesis patient (a disease known to be associated with HTLV-I) misdiagnosed for multiple sclerosis (Erlich *et al.*, 1991). Indirect evidence against an implication for HTLV-I in MS is the fact that the epidemiology of HTLV-I infection was not consistent with that of MS when family studies were conducted (Haahr *et al.*, 1991b), in which no common intrafamilial pattern of MS and HTLV-I infections was found in 79 familial cases of MS.

A few of the PCR studies of HTLV-I revealed that sequences related to HTLV-I were sometimes amplified in a proportion of both MS and control samples (Cabirac *et al.*, 1991; Dekaban and Rice, 1990; Fugger *et al.*, 1990). This was particularly noticeable when nested PCR was being employed (Prayoonwiwat *et al.*, 1991) presumably because of the low numbers of molecules it detects, which may explain why other studies failed to

reveal the presence of such sequences (Rozenberg *et al.*, 1991). Endogenous retroviruses are suspected of being the cause of these sporadic signals, further complicating the task of seeking a relationship between new retroviruses and MS using PCR technology.

The other PCR study of viruses in multiple sclerosis reported to date is that of Godec *et al.* in which tissue from active plaques of 19 multiple sclerosis patients was analyzed for the presence of the genomes of measles, mumps and rubella viruses. No viral genomes were detected in 19 multiple sclerosis brains (personal communication).

No PCR study of human coronaviruses in multiple sclerosis tissues has yet been conducted nor has an attempt been made to detect HCV-229E RNA by hybridization.

MATERIALS AND METHODS

1. Cells and virus

The human embryonic lung cell line L132 was employed for the propagation of HCV-229E as a source of viral RNA for use as a positive control in the PCR assays. Mock-infected cell cultures were employed as a source of RNA for negative controls. Both were obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.). Cells were grown at 37°C in Earle's minimal essential medium: Hank's M199 (1:1, v/v) supplemented with 10% (v/v) fetal bovine serum (FBS), 0.13% (w/v) sodium bicarbonate and 50 μg/mL gentamycin (Gibco Canada, Burlington, Ontario, Canada). A preparation of genomic OC43 RNA was kindly provided by Dr. Serge Dea (Institut Armand-Frappier, Laval, Québec, Canada).

Cell line L132 was grown in 75 cm^2 polystyrene culture flasks (Corning Glass Works, Corning, N.Y., U.S.A.) for no more than 35 passages. After trypsinization, cells were seeded at a concentration of 150,000 cells/mL in 14 cm diameter Petri dishes (Flow, Mississauga, Ontario, Canada). The cells were incubated at 37°C in a humidified atmosphere of 5% (w/v) CO_2.

1.1 Viral propagation

The fifth passage of plaque-purified HCV-229E was used to infect the approximately 75% confluent L132 cells at a multiplicity of infection of 0.01. The viral inoculum was suspended in three mL maintenance medium containing Eagle's

minimal essential medium: Hank's M199 (1:1, v/v), supplemented with 1% (v/v) fetal bovine serum, 0.13% (w/v) sodium bicarbonate and 50 μg/mL gentamycin, and left in contact with the cells for two hours at 33°C, with gentle rocking at intervals of 10 min. An additional 12 mL maintenance medium was added and the cells were incubated for another 18 to 20 hours at 33°C, 5% (v/v) CO_2. The medium was aspirated from the monolayer in order to proceed with the extraction of total cellular RNA.

1.2 Clinical specimens

Frozen tissues from the central nervous system of multiple sclerosis patients and controls were obtained from a number of sources (Table 2). The Brain Bank (Douglas Hospital, Montréal, Québec, Canada) provided tissues from five cases of multiple sclerosis, five normal brains, four brains from patients of Alzheimer's disease and from two patients of other neurological diseases: one of subacute meningo-encephalitis and one case in which signs of ischemic vascular disease had been observed at autopsy. All diseases mentioned are based on confirmed neuro-pathological autopsy reports. One specimen of white matter was collected from each of the control brains of a minimum size of 50 mg. From the brains of multiple sclerosis patients, a specimen of tissue from a visible plaque and two specimens of

TABLE 2

CNS TISSUE SPECIMENS

PATIENT	PLAQUE	WHITE MATTER	GREY MATTER
MS 1	1	1	
MS 2	1	2	
MS 3	1	1	
MS 4	2		
MS 5	2		
MS 6	2	3	
MS 7	1	1	1
MS 8	1	1	1
MS 9	1	1	1
MS 10	1	1	1
MS 11	1	1	1
NORMAL 1-5		1 EACH	
ALZHEIMER'S 1-4		1 EACH	
SUBACUTE MENINGO-ENCEPHALITIS		1	
ISCHEMIC VASCULAR DISEASE		1	

normal-appearing white matter were taken from each brain when possible.

Additional specimens of CNS tissues from MS patients were provided by Drs. Vincent Morris and George Ebers (University of Western Ontario, London, Ontario, Canada) and Dr. George P. A. Rice (University Hospital, London, Ontario, Canada). Plaque tissue from the left hemisphere, the optic nerve and the cervical cord and normal appearing white matter from the left hemisphere and the cervical cord were received, all from the same patient, from Drs. Morris and Ebers. One specimen of gray matter, one of normal-appearing white matter and one of plaque tissue from each of five MS brains were provided by Dr. Rice. After RNA extraction, the RNA for each CNS specimen was tested by RT-PCR for the presence of myelin basic protein mRNA for inclusion in the study. Specimens were coded and analyzed without knowledge of the disease of the patient from which they came to insure that they were processed in a blind manner.

1.3 Isolation of peripheral blood lymphocytes

Ten to fifteen mL whole blood from each volunteer was collected in heparinized tubes. Ten mL of blood was layered onto five mL Ficoll Hypaque (Pharmacia, Baie d'Urfé, Québec, Canada) and spun for 20 minutes at 2,500 rpm in a J6 clinical centrifuge (Beckman, Montreal, Canada). The resulting layer of

peripheral blood lymphocytes was aspirated with a sterile Pasteur pipette and transferred to a sterile, 1.5 mL Eppendorf tube. The cells were resuspended in sterile phosphate buffered saline (Gibco Laboratories, N.Y., U.S.A.) and the tubes spun for one minute at 10,000 *g*. The supernatants were removed, the cell pellets were resuspended in phosphate buffered saline and spun a second time. The supernatants were removed and the tubes were placed immediately at -70°C until the RNA was to be extracted. PBL RNA was tested for the presence of human Γ-actin mRNA to verify the quality of the RNA extracted.

2. Isolation of RNA

2.1 Preparation of materials for RNA extraction and RT-PCR analysis

To denature ribonucleases, glassware used for the preparation of solutions for RNA extraction was baked for 2 hours at 250°C. Re-usable plasticware such as stirring bars and homogenizers were washed in 10% (w/v) SDS, rinsed with DEPC-treated water and autoclaved for one hour at 121°C and 2 kg/cm^2 pressure. To avoid possible contamination with PCR products, Eppendorf tubes and disposable pipette tips were autoclaved for one hour, after siliconization. The extraction of RNA from clinical specimens, the preparation of PCR reagents and the execution of the reverse-transcription polymerase chain reaction experiments were performed in a laboratory separate

from the one in which human coronaviruses were being cultured and studied. RT-PCR was executed using Microman positive displacement pipettes (Gilson, Villiers de Bel, France).

2.2 RNA Extraction

For the extraction of clinical specimens, the guanidine isothiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987) was modified so that all manipulations were carried out in 1.5 ml Eppendorf tubes, as follows: 50 to 300 mg of CNS tissue was excised from frozen clinical specimens and immersed in 0.5 mL of solution D which contained the strong ribonuclease inhibitor, guanidine isothiocyanate, 4 M, sodium citrate 25 mM (pH 7.0), 0.5% sarcosyl and 0.72% (v/v) ß-mercaptoethanol, prepared not more than 30 days previously. CNS tissues were homogenized with plastic Eppendorf homogenizers (Kontes Scientific Glassware/Instruments/Mandel Scientific, Guelph, Ontario, Canada). Alternatively, 0.5 mL solution D was added to roughly 10^6 frozen peripheral blood lymphocytes, stored as described. Subsequently, the following solutions were added to each tube with agitation after each addition: 0.05 mL 2 M sodium acetate (pH 4.0), 0.5 mL water-saturated phenol and 0.1 mL chloroform. After the addition of chloroform, the tubes were shaken for 10 seconds and incubated on ice for 10 min. They were then centrifuged at 10,000 *g* for 15 min in a microfuge (Brinkmann Instruments, Westbury, New York, U.S.A.).

The aqueous phase was transferred to a fresh tube and the contents were mixed with 1 mL 95% (v/v) ethanol. The tubes were incubated for a minimum of 1 hour at -20°C, and centrifuged for 15 min at 10,000 *g*. The supernatants were decanted and 0.15 mL of solution D were added to resuspend the pellets. To this, 0.3 mL of 95% (v/v) ethanol were added, the contents of the tubes were thoroughly mixed and incubated for a minimum of 1 hr at -20°C. The tubes were then centrifuged for 15 min as described above, the supernatants removed and the pellets were washed by adding 0.2 mL 70% (v/v) ethanol, and centrifuging 10 min as described previously. The pellets were dried either by centrifugation for 5 min in a SpeedVac Concentrator (Savant, Farmingdale, New York, U.S.A.) or by air-drying on ice for 30 min. The pellets were suspended in 40 μl DEPC-treated water by incubating on ice for 15 min and stored frozen at -70°C.

Pronase digestion for the extraction of RNA was carried out in 400 μl buffer containing 50 mM Tris-HCl (pH 8.5), 1 mM EDTA, 0.5% (v/v) Tween 20, 1 mg/ml Pronase (Boehringer-Mannheim, Montréal, Québec, Canada) and 20 mM vanadyl ribonucleoside complexes (VRC). Samples were incubated at 55°C for three hours or at 37°C for 18 hr with continuous rocking on an Adams Nutator (Becton Dickinson, Parsipany, New Jersey, U.S.A.). They were then extracted once with an equal volume of phenol and then with an equal volume of chloroform. 0.1 mL of

0.5 M EDTA (pH 8.0) was added and the mixture incubated on ice for 15 min to remove the VRC. An additional extraction was then performed with an equal volume of chloroform. The nucleic acid was precipitated with 1/10 volume of 3 M sodium acetate (pH 6.0) and 1 mL ethanol 95% (v/v) for at least one hour at -20°C. The tubes were spun down at 7250 *g* for 20 min, washed with 70% (v/v) ethanol and dried 5 min in the SpeedVac Concentrator. The pellet was resuspended in sterile, deionized water on ice for 15 min.

For comparison, RNA from cultured L132 cells was isolated by ultracentrifugation through a cesium chloride gradient, as follows. Cells were propagated in Petri dishes 14 cm in diameter. The medium was aspirated from the dishes and the monolayers were rinsed twice with sterile PBS. 3.5 mL GIT containing 4 M guanidine isothiocyanate and 2.4 mM sodium acetate (pH 6.0) was added to the Petri dishes and the cells were taken up in the solution with a rubber policeman. The solution was layered onto 5.7 M cesium chloride, 2.4 mM sodium acetate (pH 6.0) in Ultraclear ultracentrifugation tubes (Beckman/Fisher, Montréal, Québec, Canada). The tubes were placed in a Beckman SW41 swinging bucket rotor and spun at 38,000 rpm at 20°C for 19 hr in a Beckman ultracentrifuge. Afterwards, all but roughly 400 µl solution was removed from the tubes and discarded. The tops of the tubes were cut off

approximately 1.5 cm from the bottom. The bottoms of the tubes were then inverted on absorbent paper and left to drain completely for 5 min. Each pellet was resuspended in 300 µl 0.3 M sodium acetate (pH 6.0), transferred to a 1.5 mL Eppendorf tube and 750 µl 95% (v/v) ethanol was added. The tubes were inverted several times and placed at -20°C for one hour. They were centrifuged at 7250 *g* for 20 min, washed with 70% (v/v) ethanol and dried in a SpeedVac Concentrator for 5 min. The pellets were resuspended in sterile, deionized water and an aliquot was removed for estimating the yield by spectrophotometry.

2.2.1 Yield of RNA and estimation of quality

The quantity of RNA recovered was estimated by measuring the optical density in a Gilford 2400S spectrophotometer at 260 nm. An optical density of 1.0 was considered to represent a concentration of RNA of 40 µg/mL. To evaluate quality in terms of a lack of protein and phenol contamination, the ratio of the optical densities at 260 nm to 280 nm was calculated (Sambrook *et al.*, 1989) and a value of >1.6 was considered to represent acceptable quality, in spite of the presence of protein at this ratio.

2.3 Formaldehyde-agarose gels for RNA analysis

The gel apparatus (International Biotechnologies, Inc., New Haven, Connecticut, U.S.A.) was washed with 10% (w/v) SDS and rinsed with deionized water. A 10X MOPS buffer solution was prepared containing 0.2 M MOPS, 50 mM sodium acetate and 10 mM EDTA (pH 5.4). A 1% (w/v) agarose gel was prepared by mixing 10 mL buffer, 84.6 mL water and 1 g agarose and heating 5 minutes in a microwave oven. The gel was cooled to 50°C, 5.4 mL formaldehyde (36.5-38%) and 0.5 μg/mL ethidium bromide were added under a chemical hood, the gel was mixed thoroughly and poured into the gel mold. The gel was left for twenty minutes to harden. The RNA samples to be analyzed were completely dried in the SpeedVac Concentrator and resuspended in 20 μl of loading buffer containing 1X MOPS buffer, 50% (v/v) formamide, 17% (v/v) formaldehyde (36.5-38% (v/v)), 6% (v/v) glycerol and 0.05% (v/v) of a saturated solution of bromophenol blue. The samples were allowed to resuspend 10 minutes on ice, heated at 85°C for 2 minutes and cooled for five minutes on ice before loading. The gel was migrated for 2 hours at 3-4 volts/cm. RNA was visualized under ultra violet light.

3. Reverse transcription polymerase chain reaction (RT-PCR)

Master mixes of both the reverse transcription and the polymerase chain reaction mixtures were prepared prior to use

and aliquoted in 0.5 mL Eppendorf tubes. The PCR reaction mixtures were overlaid with 100 µl mineral oil (Sigma).

3.1 Primers, target sequences and oligonucleotide probes

All target sequences were roughly 300 bases in length. The primer pairs selected were verified for the absence of secondary structure and contiguous complementarity between primer pairs of more than three nucleotides at the 3' ends using the Oligotest (2.0) program (Beroud *et al.*, 1990). All contained a percentage of guanosine and cytidine (GC content) of 50 to 60. Human MBP primers corresponded to bases 274-307 (exon 3) and were inverse and complementary to bases 550-573 (exon 7) of the human MBP gene (Streicher and Stoffel, 1989) (Fig. 3). They were 5'-AGAACTGCTCACTACGGCTCCCTG-3' and 5'-TCCAGAGCGACTATCTCTTCCTCC-3', respectively. Human Γ-actin primers corresponded to bases 1358-1381 (exon 3) and were inverse and complementary to bases 1712-1735 (exon 4) of the human Γ-actin gene (Erba *et al.*, 1988) (Fig. 3). They were 5'-GACCTGACCGACTACCTCATGAAG-3' and 5'-GGAGTTGAAGGTGGTCTCGTGGAT-3', respectively. HCV-229E primers corresponded to bases 498-521 and were inverse and complementary to bases 783-806 on mRNA 7 of this virus. They were 5'-AGGCGCAAGAATTCAGAACCAGAG-3' and 5'- AGCAGGACTCTGATTACGAGAAGG-3', respectively. A second set of primers for HCV-229E corresponded to bases 964-982 and were inverse and complementary to bases 1242-1265 on mRNA 7

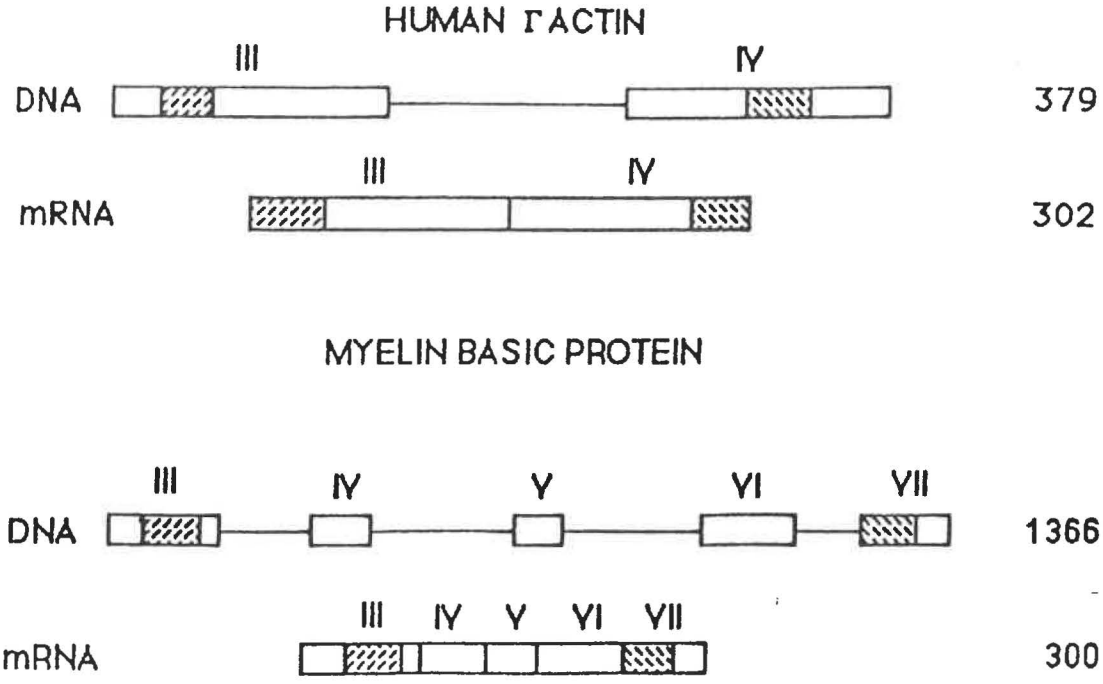
(Schreiber *et al.*, 1989). They were 5'-AACTTTGGAAGTG CAGGTGTTGTG-3' and 5'-TGGTTCAGCAGTTGCAGGTGAAGT-3', respectively. Primers for detecting HCV-OC43 corresponded to bases 215-238 and were inverse and complementary to bases 497-520 on the mRNA coding for the nucleoprotein of this virus (Kamahora *et al.*, 1989). They were 5'-CCCAAGCAAACTGCTACCTCTCAG-3' and 5'GTAGACTCCGTCAATATCGGTGCC-3', respectively.

Oligonucleotide probes 24 bases in length for hybridization with the amplification products corresponded to sequences located between each pair of primers and thus hybridized to the negative strand of the amplification product. They were 5'- ATGAAGGCAGTTGCTGCGGCTCTT-3' (bases 693-716) for primer pair #1 and 5'-GAGTCAGGCAACACTGTGGTCTTG-3' (bases 1080-1103) for primer pair #2 of HCV-229E; 5'-GATGGCAACCAGCGTCAACTGCTG-3' (bases 419-442) for HCV-OC43. For human Γ-actin and MBP RNAs, they were 5'-GAAATCGTGCGCGA CATCAAGGAG-3' (bases 1432-1455) and 5'-CCCGTAGTCCACTTCTTCAAGAAC-3' (bases 334-357), respectively.

3.2 Reverse transcription

Three μl of RNA extract was added to 17 μl of master reverse transcription mix to achieve final concentrations of 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 0.1% (v/v) Triton X-100 (*Taq* polymerase buffer, BIO/CAN, Mississauga, Ontario, Canada), 20

Figure 3: Locations of target sequences on human Γ-actin and MBP genes and mRNA. The predicted sizes of amplification products are indicated on the right.


HUMAN Γ ACTIN
III
IV
DNA
379
III
IV
mRNA
302
MYELIN BASIC PROTEIN
III
IV
V
VI
VII
DNA
1366
III
IV
V
VI
VII
mRNA
300
EXONS
INTRONS
PRIMERS

U Moloney Murine Leukemia Virus reverse transcriptase (Pharmacia, Baie d'Urfé, Québec, Canada), 40 U RNAguard (Pharmacia), 50 pmoles of both up- and downstream primers, 1.0 mM each of dATP, dCTP, dGTP and dTTP (Pharmacia), and 4.0 mM $MgCl_2$ and incubated at 37°C for 35 min. Initially, concentrations of dNTPs from 0.25 mM to 1.0 mM, of $MgCl_2$ from 2.75 mM to 6.5 mM and quantities of primers from 25 to 100 pmoles were all tested on 0.1 μg RNA from HCV-229E infected cells.

3.3 Polymerase Chain Reaction

After incubation, the entire volume of the reverse transcription reaction mixture was added to 80 μl of a PCR master mix overlaid with mineral oil. This mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 0.1% (v/v) Triton X-100, 2.5 U *Taq* polymerase (BIO/CAN), 50 pmoles up- and downstream primers, 0.25 mM each of dATP, dCTP, dGTP and dTTP (Pharmacia) and 2.4 mM $MgCl_2$. An amplification cycle of 1 min at 94°C, 2 min at 60°C and 2 min at 72°C was repeated 30 times and followed by an extension period of 7 min at 72°C in a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, Connecticut, U.S.A.). Initially, concentrations of dNTPs from 0.0625 to 0.25 mM, of $MgCl_2$ from 2.0 mM to 3.4 mM and quantities of primers from 25 to 100 pmoles were tested with 0.1 μg RNA from HCV-229E infected cells.

3.4 Analysis of PCR amplification products

Twenty μl of amplification reaction was analyzed by agarose gel electrophoresis. Subsequently a Southern blot of the gel was performed and the final result was obtained after hybridization with a ^{32}P-labelled oligonucleotide probe.

3.4.1 Agarose gel electrophoresis

Agarose gels were prepared and run in TAE buffer containing 0.04 M Tris-acetate and 1 mM EDTA (pH 8.0). The gel contained 1.5% (w/v) SeaKem agarose (FMC Bioproducts/ Mandel Scientific, Rockwood, Ontario, Canada) and 0.5 μg/mL ethidium bromide. Amplification products were migrated at 3-4 volts/cm for 2 hours. One lane was loaded with 2 μg of *Hae*III-digested pBR322 as a molecular size marker.

3.4.2 Southern blotting

Gels were denatured twice 30 min in a solution containing 1.5 M NaCl and 0.5 N NaOH. They were then neutralized in 1 M ammonium acetate and 0.02 N NaOH. The DNA was transferred onto 0.45 μm Hybond-C extra nitrocellulose membranes (Amersham, Oakville, Ontario, Canada) by capillary action overnight in the neutralization solution. Filters were baked 2 hrs at 80°C in a vacuum oven.

3.4.3 Hybridization

3.4.3.1 Preparation of oligonucleotide probes

Oligonucleotide probes were labelled with ^{32}P in a volume of 75 µl containing 30 U T4 polynucleotide kinase (Pharmacia, Baie d'Urfé, Québec, Canada), 40 pmoles oligonucleotide, 250 pmoles [Γ-^{32}P]ATP (ICN, Lisle, Illinois, U.S.A.) and 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 5 mM DTT and 1 mM spermidine. After incubating the mixture 40 min at 37°C, it was diluted with deionized, sterile water to a volume of 200 µl, 2 µl was blotted on a filter and the remainder was phenol-chloroform and chloroform extracted and used for hybridization the same day. The filter containing the 2 µl aliquot was transferred to a bath of 10% (w/v) trichloroacetic acid, and to two subsequent baths of 70% (v/v) ethanol. After drying, it was immersed in Aquasol-2 (DuPont, Boston, Massachussets, U.S.A.) scintillation cocktail and the ß radiation counted.

3.4.3.2 Hybridization

Blot filters were placed in polyethylene bags, immersed in 5 mL of hybridization solution containing 0.75 M NaCl, 0.075 M sodium citrate, 5 mg Ficoll 400, 5 mg polyvinyl pyrrolidone, 5 mg bovine serum albumin, Fraction V), 0.05% (w/v) sodium pyrophosphate, 100 µg/mL sonicated salmon sperm DNA and 0.2% (w/v) SDS (pH 7.0). This amounted to a final concentration of

Denhardt's solution of 5X. The bags were sealed and placed to pre-hybridize in a 37°C water bath for a minimum of 1 hr. The labelled oligonucleotide was added to an additional 5 mL of hybridization solution and subsequently added to the bag containing the filter, which was then heat-sealed. The bags were placed in a 50°C water bath and agitated for 16 hrs. The filters were then washed thrice for 15 min at room temperature and for 20 min at 60°C in a solution containing 0.75 M NaCl, 0.075 M sodium citrate, 0.05% (w/v) pyrophosphate, (pH 7.0). They were wrapped in Saran wrap and exposed to X-ray film with intensifying screens (Kodak, Rochester, New York, U.S.A.) for 48 to 96 hrs at -70°C.

4. Estimation of the level of detectability of the RT-PCR assay

4.1 Cloning of the amplification product of the RT-PCR to detect HCV-229E

4.1.1 Preparation of amplification product for cloning

Amplification products from three previous RT-PCR assays were pooled in a total volume of 200 µl. They were extracted once with phenol-chloroform and twice with chloroform. Then they were precipitated with a 1/10 volume of 3 M sodium acetate and three volumes of ethanol for one hour at -20°C. They were washed with 70% (v/v) ethanol, dried 5 min in a SpeedVac Concentrator and resuspended in 40 µl deionized, sterile water

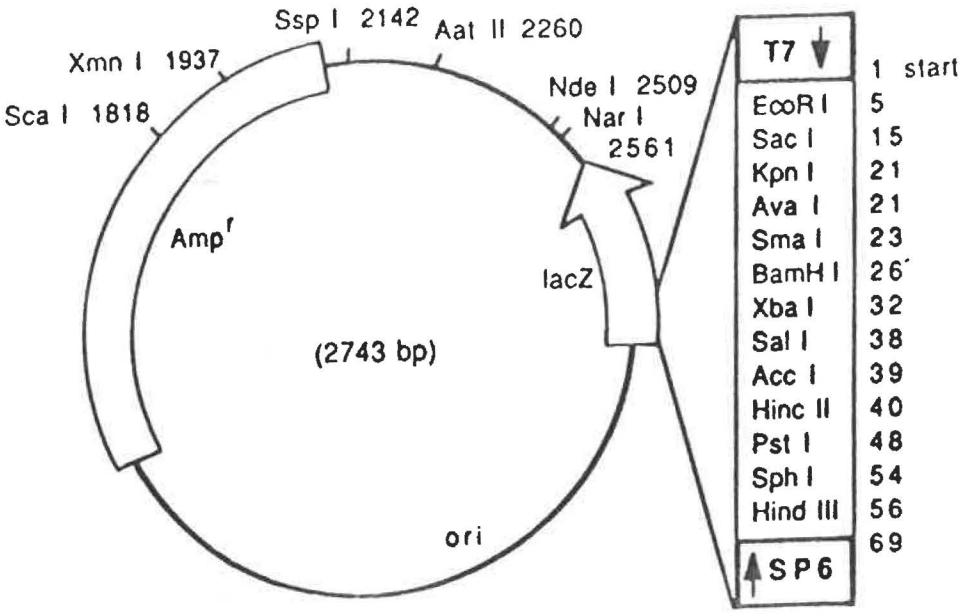
for one hour on ice. One fourth, roughly 2.5 μg, were incubated with 10 U Klenow fragment of DNA polymerase I (Pharmacia) to fill in overhangs left by *Taq* polymerase during amplification. In addition, the reaction mixture contained 0.05 mM dNTPs, 0.05 M Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM DTT and 5 μg/ml BSA (Sigma, St. Louis, Missouri, U.S.A.) and was incubated 30 min at 37°C. The reaction mixture was used directly for ligation.

4.1.2 Ligation

The circular form of the pGEM®-3Z plasmid (Fig. 4) was prepared for ligation by digestion with *Sma*I, in order to clone at that site. A reaction mixture containing 1x *One Phor All Plus* buffer (Pharmacia), containing 10 mM Tris-acetate (pH 7.5), 10 mM magnesium acetate and 50 mM potassium acetate, 4.5 μg pGEM -3z vector (Promega/Fisher, Montreal, Québec, Canada) 20 U *Sma*I (BRL), in a total volume of 40 μl was incubated 2 hours at 30°C.

Blunt-end ligation was performed for 18 hrs at 12°C using 80 ng of amplification product and 100 ng of pGEM®-3z plasmid, both prepared as described above, giving a molar ratio of approximately 8:1 of insert to vector. The reaction mixture also contained 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 20 mM DTT, 1 mM ATP, 5 μg/ml BSA and 10U T4 DNA ligase (Pharmacia, Baie

Figure 4: Diagram of the pGEM®-3Z plasmid


Ssp I 2142
Aat II 2260
Xmn I 1937
Sca I 1818
Nde I 2509
Nar I
2561
Amp^r
lacZ
(2743 bp)
ori
T7
1 start
EcoR I 5
Sac I 15
Kpn I 21
Ava I 21
Sma I 23
BamH I 26
Xba I 32
Sal I 38
Acc I 39
Hinc II 40
Pst I 48
Sph I 54
Hind III 56
69
SP6

d'Urfé, Québec, Canada). The ligation mixture was used directly in transformation.

4.1.4 Competent cells

Competent *E. coli* JM105 cells were prepared as follows (D. Hanahan, 1985): fresh colonies were grown on SOB agar containing 2% (w/v) bacto tryptone (Gibco Canada, Burlington, Ontario, Canada), 0.5% (w/v) bacto yeast extract (Gibco), 10 mM NaCl, 2.5 mM potassium chloride and 1.5% (w/v) Bacto agar (Gibco). Sterile magnesium chloride and magnesium sulfate solutions were added just prior to pouring the plates to achieve final concentrations of 10 mM each. Several colonies were dispersed in 30 mL SOB medium, which contained the same components as SOB agar except for the Bacto agar, in a one liter Erlenmeyer flask loosely stoppered with sterile cotton batting. The flask was incubated at 37°C with rotation at 175 rpm (LabLine Incubator Shaker, Fisher, Montreal, Québec, Canada) until the optical density at 600 nm reached between 0.3 and 0.6. The culture was chilled on ice for 15 min. The cells were pelleted at 1000 *g* for 15 min, the supernatant was discarded and the tubes inverted on absorbent paper to remove all excess liquid. The cell pellet was resuspended in 10 mL RF1 solution containing 100 mM rubidium chloride, 50 mM manganese chloride, 30 mM potassium acetate, 10 mM calcium chloride and 15% (v/v) glycerol (pH 5.8), which had been sterilized by

filtration through a 0.22 μm membrane. The cells in suspension were incubated for 20 min on ice. They were pelleted by centrifugation at 1000 *g* for 15 min and resuspended in 2.5 mL RF2 containing 10 mM MOPS, 10 mM rubidium chloride, 75 mM calcium chloride and 15% (v/v) glycerol and incubated on ice for 15 min. They were then mixed with an equal volume of sterile 50% (v/v) glycerol, and 300 μl were aliquoted in Eppendorf tubes and placed in a -70°C freezer. They were stored frozen at -70°C until use within three months.

4.1.4 Transformation

Frozen competent cells were thawed at room temperature and 200 μl was immediately mixed with 8 μl of the ligation reaction, incubated 45 min on ice and 2 min at 42°C in Eppendorf tubes. One mL of LB broth (Gibco) was added and the tubes were incubated at 37°C for 45 min. Approximately 100 μl of this preparation was plated on LB agar containing 100 μg/mL ampicillin, 3.3 mM IPTG and 0.01% (w/v) X-gal (Clontech/BIO/CAN Scientific, Inc., Mississauga, Ontario, Canada) in 3 mL of 0.7% (w/v) top agar overlaid on an LB agar base. A negative control of cells transformed with non-recombinant plasmid was prepared in parallel. The plates were incubated overnight at 37°C.

4.1.5. Colony hybridization of recombinant clones

White, transformed recombinant colonies were re-inoculated onto LB agar containing 100 μg/mL ampicillin along with several blue colonies from the control plate transformed with non-recombinant plasmid. The new plate was incubated at 37°C for 28 hours. A nitrocellulose filter was cut to the size of the Petri dish and wetted on a fresh LB plate. It was then placed on the surface of the plate containing the recombinant bacteria for several minutes. Whatman 3MM filter paper was wetted with a denaturing solution of 0.5 M NaOH and 1 M Tris-HCl (pH 7.5) and an additional sheet was wetted with a neutralizing solution of 1.5 M NaCl and 0.5 M Tris-HCl (pH 7.5). The nitrocellulose filter was placed for three minutes on the first sheet and then three minutes on the second. It was allowed to dry and baked for one hour at 80°C in a vacuum oven. It was hybridized with the HCV-229E internal oligonucleotide probe as described in section 2.4.3. After washing, it was exposed to X-ray film (Kodak) for 30 min at room temperature.

4.1.6 Mini-preparation of recombinant plasmid

Three of five colonies giving positive hybridization signals were inoculated in 2 mLs of LB broth containing 75 μg/mL ampicillin and incubated at 37°C with 200 rpm rotation overnight. Mini-preps were prepared according to the boiling

method (Sambrook *et al.*, 1989), as follows. One mL of each was transferred to a 1.5 mL Eppendorf tube and centrifuged for 2 min at 7250 *g*. The supernatants were removed by aspiration, the pellets were resuspended in 200 µL STET (0.1 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and 5% (v/v) Triton X-100) and incubated for 20 min on ice. Then 20 µL of 5 mg/mL lysozyme (Boehringer Mannheim, Laval, Québec, Canada) was added to each and the tubes were placed in boiling water for 30 sec. The tubes were then centrifuged at 7250 *g* for 20 min and the pellets were removed with toothpicks and discarded. The remaining supernatants were mixed with equal volumes of isopropanol and precipitated at -20°C for 30 min. The DNA was pelleted by centrifugation at 7250 *g* for 15 minutes. After decanting, the pellets were washed with 70% (v/v) ethanol, dried 5 min in a SpeedVac Concentrator and left to resuspend on ice for 2 hours in 60 µL sterile, deionized water.

4.1.7 Restriction enzyme digestion of recombinant plasmids

The sequence of the amplification target and the nucleotide map of the pGEM® -3Z vector were analyzed to determine appropriate restriction enzyme sites whose cleavage would result in fragments of sizes that would reveal the directionality of the inserted PCR amplification product.

Five μL of each mini-prep was digested with 5 U *Pvu*II (BRL, Gaithersburg, Maryland, U.S.A.) and 5 U *Eco*RI (BRL) in *One Phor All Plus* buffer for 3 hours at 37°C. The resulting fragments were visualized on an agarose gel using 3% (w/v) NuSieve and 1% (w/v) SeaKem (FMC bioproducts) in TAE buffer containing 0.5 μg/mL ethidium bromide.

4.1.8 Plasmid purification

One of the clones was inoculated in 2 mL of LB broth containing 75 μg/mL ampicillin and incubated for 8 hours at 37°C and 175 rpm. Two mL was then transferred to an Erlenmeyer flask containing 25 mL of the same broth, and it was incubated overnight under the same conditions. The plasmid was isolated using a Plasmid quik™ purification kit (Stratagene, La Jolla, California, U.S.A.). Briefly, 20 mL of the overnight culture was spun down at 2000 *g* for 10 min. The supernatant was removed and the pellet was resuspended in 1 mL complete lysis buffer containing 8% (w/v) sucrose, 10 mM Tris-HCl (pH 8.0), 50 mM EDTA, 0.5% (v/v) Triton X-100, 1 mg/mL lysozyme and 17% (v/v) RNace-it™ cocktail. The suspension was transferred to 2 Eppendorf microcentrifuge tubes (600 μl/tube) and these were placed in a boiling water bath for 30 seconds. They were spun at 7250 *g* in a microfuge for 20 min. The pellet was removed with a toothpick and discarded. To each tube, 500 μl loading buffer containing 1 M NaCl, 5 mM Tris-HCl (pH 8.0) and 0.1 mM

EDTA was added. The tubes were then placed in a boiling water bath for 5 minutes and then immediately on ice. The chilled sample from both tubes was loaded into the buffer head in the column of resin provided with the kit. The fluid was pushed through the column at a rate close to 1 drop/second, collected in fresh Eppendorf tubes and precipitated with an equal volume of isopropanol. Then, the tubes were spun at 7250 *g* for 10 min, the pellets transferred to a single tube, washed twice with 70% (v/v) ethanol and dried five min in a SpeedVac Concentrator. The pellet was suspended in 50 µl sterile, deionized water for one hour on ice.

4.2 *In vitro* transcription

4.2.1 Linearization of plasmid

Forty µL of plasmid suspension was incubated with *One Phor All Plus* buffer (Pharmacia) and 20 U *Hin*dIII for 3 hours at 37°C. After digestion, the reaction mixture was diluted to 200 µL and extracted once with PCI and twice with chloroform and precipitated with 1/10 volume 3 M sodium acetate, pH 6.0 and three volumes ethanol overnight at -20°C. The DNA was pelleted at 7250 *g*, the pellet washed with 70% (v/v) ethanol, dried 3 min in a SpeedVac Concentrator and resuspended for one hour in 30 µL sterile, deionized water on ice.

4.2.2 *In vitro* transcription of the RT-PCR product of HCV-229E

Roughly 5 μg of linearized plasmid was transcribed *in vitro,* taking advantage of the T7 promoter site flanking the multiple cloning site (Fig. 4). Transcription buffer contained final concentrations of 20 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 1 mM spermidine and 25 mM NaCl. Unlabelled ribonucleotides ATP, GTP and UTP were included at concentrations of 0.4 mM each. The sole source of CTP was 3.0 μM [α-^{32}P]CTP (ICN) with a specific activity of 650 Ci/mmole. Additionally, 30 mM dithiothreitol, 20 U T7 RNA polymerase (Stratagene, La Jolla, California, U.S.A.) and 2 U RNA Block II (Stratagene) were included and the reaction was incubated for one hour at 37°C. Then, 20 U ribonuclease-free DNAse (Stratagene, La Jolla, California, U.S.A.) was added and incubated another hour at 37°C. The transcription reaction was diluted to a total volume of 200 μl with sterile, deionized water, extracted once with PCI and twice with chloroform. Then, the RNA was precipitated with a 1/10 volume of 3 M sodium acetate and three volumes of ethanol for one hour at -20°C. After pelleting, washing and drying as described previously, it was suspended in 20 μl DEPC-treated water. Five μl of this suspension was diluted to 125 μl and an aliquot of three μl was used for scintillation counting. Three μl of a 1/10 dilution of [α-^{32}P]CTP was also counted for inclusion in the final calculation of moles of CTP incorporated

during transcription. Tenfold serial dilutions (10 µl transcript dilution + 90 µl DEPC-treated water) of transcript were tested by RT-PCR.

5. Cloning of HCV-OC43 amplification product

One µL of the HCV-OC43 RT-PCR amplification product which contained a fragment of roughly 300 base pairs was re-amplified using the PCR method, described in section 2, in a total volume of 100 µl, using the same pair of primers as for HCV-OC43. One µl of this amplification product was diluted ten-fold, and one µl of this dilution was used in the ligation reaction.

5.1 Ligation

The TA Cloning™ System (Invitrogen Corporation, San Diego, California, U.S.A.) was employed to clone the PCR product. Fifty ng of pCR™ 1000 plasmid, roughly 10 ng of PCR amplification product as prepared above, 1X ligation buffer (Invitrogen), 5 U T4 DNA ligase (Pharmacia) in a total of 11 µl was incubated at 12°C overnight. This reaction mixture was used directly in transformation.

5.2 Transformation

One 50 µl vial of frozen, competent *E. coli* cells (INVαF', Invitrogen™) was thawed, 2 µl of 0.5 M ß-mercaptoethanol was added immediately and the tube was mixed by gentle tapping. One

μl of the ligation reaction mixture was added, the tube tapped gently and incubated on ice for 30 min. Then the tube was incubated for 60 seconds in a 42°C water bath. The cell suspension was mixed with 450 μl SOC medium containing 2% (w/v) bacto-tryptone, 0.5% (w/v) bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM each magnesium chloride and magnesium sulfate and 20 mM glucose. The vial was incubated for one hour at 37°C and 225 rpm. An LB agar plate containing 50 μg/mL kanamycin was prepared by spreading 25 μl of a 40 mg/mL solution of X-gal which was allowed to diffuse into the agar for one hour. The transformed cells were placed on ice and 25 μl were plated on the LB agar prepared as described. The plate was inverted and incubated overnight at 37°C.

5.3 Mini-preparation of recombinant plasmids

Colonies of 15 recombinant clones and one non-recombinant clone were each inoculated in two mL LB broth containing 50 μg/mL kanamycin and incubated overnight at 37°C with shaking at 175 rpm. The plates were incubated at 37°C for several hours to allow the colonies to grow back. Fifteen of the original 16 broth cultures were used to make the mini-preps by the alkaline lysis method (Sambrook *et al.*, 1989), as follows: 1.5 mL of each broth was transferred to an Eppendorf tube and centrifuged one minute at 12,300 *g* in an Eppendorf 5415C microfuge. The supernatants were removed by aspiration and the pellets were

resuspended in 100 µl of an ice cold, sterile solution containing 50 mM glucose, 25 mM Tris-HCl (pH 8.0) and 10 mM EDTA. Then 200 µl of freshly prepared 0.2 N NaOH, 1.0% (w/v) SDS was added to each and the tubes were inverted five times and stored on ice. An ice-cold solution containing 3 M potassium and 5 M acetate was added in aliquots of 150 µl to each tube, the tubes were vortexed and incubated on ice for 5 min. They were then centrifuged at 12,300 *g* for 5 min and the supernatants were extracted once with phenol-chloroform. The aqueous phase was precipitated with 2 volumes ethanol at room temperature for 2 min, centrifuged for 5 min at 12,300 *g*. The supernatants were removed by aspiration and the tubes inverted five minutes. The pellets were rinsed with 200 µl 70% (v/v) ethanol and then allowed to air-dry for ten minutes. The pellets were resuspended in 50 µl sterile, deionized water containing 2.5 µg ribonuclease A (Pharmacia) and allowed to stand 45 min.

5.4 Analysis of recombinant clones by restriction enzyme cleavage

Five µl of each mini-prep was digested with 10 U *Hin*dIII, and separately with 10 U each of *Hin*dIII and *Eco*RI (Pharmacia) in a total of 20 µl of *One Phor All Plus* buffer. The reactions were incubated for 3 hours at 37°C. Each reaction mixture was then loaded onto 1.5% (w/v) agarose gels prepared as described

in section 2.4. The DNA was transferred onto nitrocellulose and the filter was baked and hybridized with a labelled oligonucleotide corresponding to the first HCV-OC43 PCR primer, all according to the protocols described in section 2.4.

5.5 Preparation of DNA for sequencing

Five clones giving positive hybridization signals and the clone of non-recombinant plasmid were propagated overnight in 50 mL of LB containing 50 μg/mL of kanamycin. They were centrifuged 15 min at 2500 *g* at 4°C. The pellets were resuspended in 10 mL of a solution containing 0.1 M NaCl, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. They were then centrifuged for 15 min at 2500 *g*. The pellets were suspended in 2 mL of a cold solution of 50 mM glucose, 25 mM Tris-HCl (pH 8.0) and 10 mM EDTA, vortexed, and incubated on ice for 5 min. Four mL of a freshly prepared solution of 0.2 M NaOH and 1% (w/v) SDS were added to each, the tubes were gently mixed by inversion and incubated at room temperature for 10 min. Two mL of cold 3 M potassium and 5 M acetate were added, the solutions were mixed gently and incubated on ice for 10 min. They were centrifuged for 15 min at 2250 *g* and the supernatants were filtered by gravity through a 67 μm mesh polyester tissue (B.& S.H. Thompson, Ltée, Mount Royal, Québec, Canada). Four mL isopropanol was added to each tube, and the DNA was precipitated at room temperature for 10 min. They were

centrifuged for 15 min at 4000 *g*, the pellets suspended in 300 µl 70% (v/v) ethanol, transferred to Eppendorf tubes and pelleted 10 min at 12,300 *g*. The pellets were dried in a SpeedVac Concentrator for 5 min and resuspended in 300 µl 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (TE). An equal volume of ice cold 5 M lithium chloride was added to each tube, the tubes were vortexed and centrifuged for 10 min at 12,300 *g*. The supernatants were precipitated with an equal volume of isopropanol, centrifuged at 12,300 *g* for 10 min. The supernatants were decanted, the pellets rinsed with 70% (v/v) ethanol and dried 5 min in a SpeedVac Concentrator. The pellets were dissolved in 500 µl TE containing 20 µg/ml ribonuclease A and incubated 30 min at room temperature. An equal volume of a solution of 1.6 M NaCl and 13% (w/v) polyethylene glycol 8000 was added to all the tubes, which were vortexed and centrifuged for 10 min at 12,300 *g*. The supernatants were discarded and the pellets suspended in 200 µl TE. The suspensions were extracted once with phenol, once with phenol-chloroform and once with chloroform. They were then precipitated with 1/10 volume 3 M sodium acetate and two volumes ethanol and kept 10 min on ice. Finally, they were centrifuged for 10 min at 12,300 *g* and the pellets were washed with 70% (v/v) ethanol. The pellets were dried for 5 min in a SpeedVac Concentrator and resuspended in sterile, deionized water.

5.6 Sequencing

Three μg of each plasmid preparation was suspended in 10 μl of sterile, deionized water and delivered to the Service de Séquençage, Institut Armand-Frappier, where they were sequenced by the dideoxy chain termination method with incorporation of [^{35}S]dATP (Sanger *et al.*, 1977). Briefly, double-stranded DNA was denatured in an alkaline solution, neutralized with ammonium acetate and ethanol precipitated. Sequencing was performed using the Pharmacia sequencing kit with a forward M13 universal primer and T7 DNA polymerase. Sequences were analysed on polyacrylamide/urea gels and the gels were exposed to Kodak X-ray film for 72 hours at -70°C.

6.0 Sequence analysis

The GeneWorks program, version 2.0 (IntelliGenetics, Mountain View, California, U.S.A.) was utilized to determine the degree of nucleotide and amino acid identity of the amplified sequence with the target sequence on HCV-OC43.

RESULTS

1. RNA Extraction

The detection of human coronavirus RNA in clinical specimens required a relatively quick and simple method of RNA extraction. Testing of the guanidine isothiocyanate-phenol-chloroform micro-method selected was performed on cultured L132 cells that were either infected with HCV-229E or mock-infected. The yield and quality of the test extractions as compared with the standard method of extraction with guanidine isothiocyanate and isolation by ultracentrifugation through cesium chloride gradients can be seen in Table 3. The micro-method gave yields of RNA 37% lower on average than did the standard technique, and was considered to be acceptable for our purposes. The ratio of optical density at 260 nm to that at 280 nm was greater than 1.6, indicating that there was little protein contamination of the nucleic acid (Materials and Methods, section 1.6.2).

It was considered necessary to verify that the RNA extracted was not significantly degraded and this was done by two methods. The first was the visualization of ribosomal RNAs on formaldehyde agarose gels by ethidium bromide staining (Materials and Methods, section 1.7). Both 18S and 28S rRNAs were readily visible (Fig. 5). The second method of verifying the suitability of RNA for RT-PCR analysis was the amplification of cellular mRNAs expressed in the tissues to be analyzed (Materials and Methods, section 2.1). Target sequences

TABLE 3

COMPARISON OF GUANIDINE ISOTHIOCYANATE-PHENOL-CHLOROFORM RNA EXTRACTION WITH ISOLATION BY ULTRACENTRIFUGATION OF CESIUM CHLORIDE GRADIENTS

EXTRACTION METHOD	CESIUM CHLORIDE GRADIENT		MICRO-METHOD extraction #1		MICRO-METHOD extraction #2		MICRO-METHOD extraction #3	
L132 CELLS*	UI	I	UI	I	UI	I	UI	I
YIELD ($\mu g/10^7$ cells)	147	133	64	66	160	116	66	56
OPTICAL DENSITY 260/280 nm	1.97	1.9	1.67	1.73	1.75	1.83	1.82	1.74

*Cells are either uninfected (UI) or infected (I) with HCV-229E.

Figure 5: Formaldehyde gel electrophoresis of total RNA extracted from L132 cells using the guanidine isothiocyanate-phenol-chloroform extraction method.

Lane 1: ribosomal RNA from L132 mock-infected cell cultures.
Lane 2: ribosomal RNA from L132 cell cultures infected with HCV-229E.

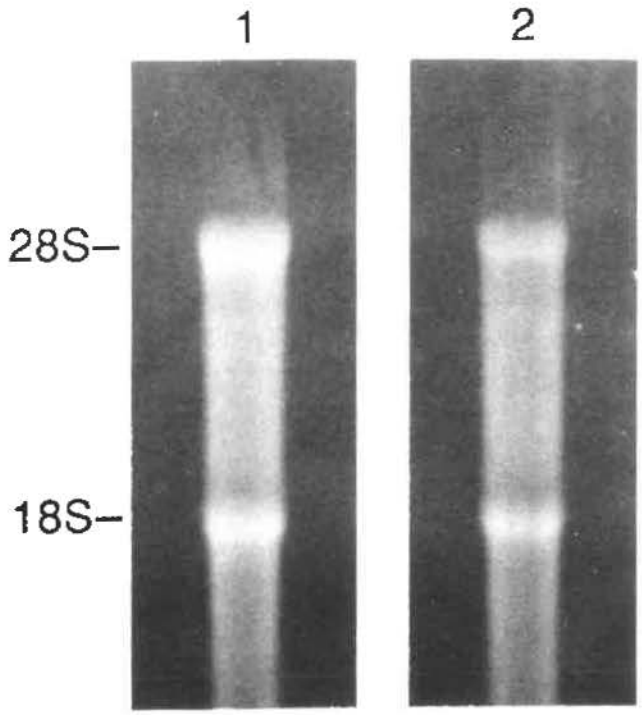
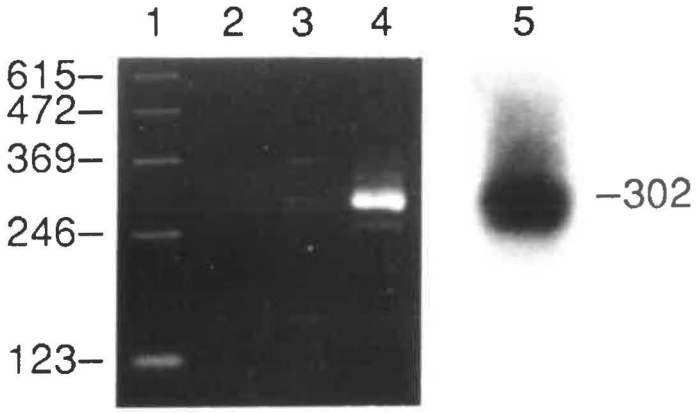

1
2
28S–
18S–

Figure 6: Reverse transcription-polymerase chain reaction to detect human Γ-actin mRNA.

Lane 1: 123 base pair ladder DNA markers.
Lanes 2-4 : Visualization on agarose gels by ethidium bromide staining of the amplification products of RT-PCR on the following templates: no RNA (2), RNA isolated from Brome Mosaic virus (3) and RNA isolated from human peripheral blood lymphocytes (4).

Lane 5: Autoradiography of the product of amplification of human PBL RNA after transfer to nitrocellulose and hybridization with a ^{32}P-labelled oligonucleotide probe specific for the target sequence on Γ-actin RNA.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615–
472–
369–
246–
123–
–302

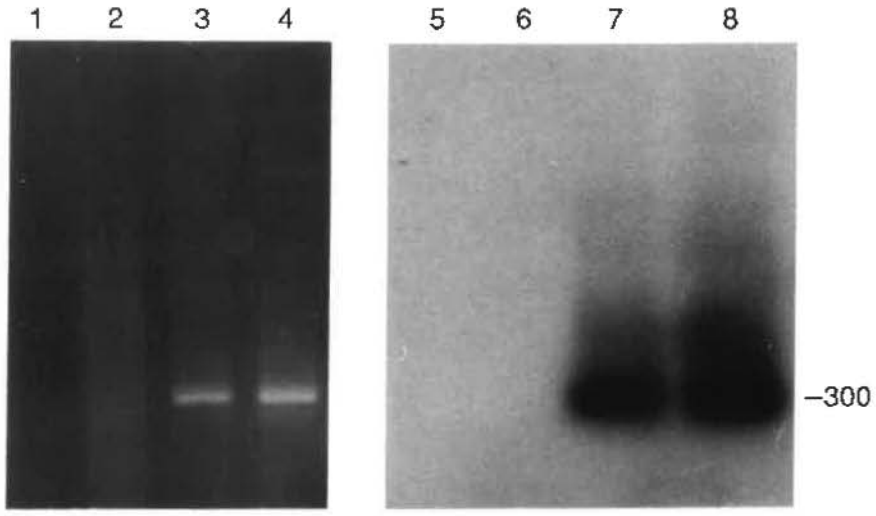
were selected on mRNA that were similar in size to the target sequences on viral RNA. Human Γ-actin is expressed in all cells and therefore a target sequence on the mRNA coding for it was selected. Human MBP mRNA is expressed in CNS white matter and the primers for MBP mRNA were used to test each RNA extraction from white matter for amplifiable RNA. In order to insure that the RNA amplified would be distinguishable from contaminating DNA, the target sequence on cellular mRNAs contained intervening introns (Fig. 3) which would result in amplification products of different sizes from mRNA and DNA. In Fig. 6, the amplification of human Γ-actin mRNA from cellular RNA extracts can be seen thus confirming the applicability of the RNA extraction method in this study. Furthermore, the absence of a band of roughly 380 base pairs, the predicted size of the amplification product of the target sequence on the gene itself indicates that little or no contaminating DNA is present using this method of extraction. Likewise, the amplification product of human MBP mRNA from human CNS white matter can be seen in Fig. 7. No band corresponding to 1366 base pairs, the predicted size of the MBP DNA amplification product, was ever observed.

At the beginning of the study, formalin-fixed brain tissue was to be analyzed for the presence of human coronavirus RNA. Pronase digestion of these tissues for RNA extraction was

Figure 7: Amplification of human MBP mRNA at two different annealing temperatures.

Lanes 1-4: Visualization of RT-PCR amplification products on agarose gels by ethidium bromide staining. Templates are no RNA (1), total RNA from L132 cells (2), RNA from human CNS white matter, annealing temperature of PCR 55°C (3) and RNA from human CNS white matter, annealing temperature of PCR 60°C (4).

Lanes 5-8: amplification products above were transferred to nitrocellulose and hybridized with a ^{32}P-labelled oligonucleotide probe specific for the target sequence on human MBP mRNA. Products hybridized are from the templates above: no RNA (5), total RNA from L132 cells (6), RNA from human white matter, annealed in PCR at 55°C (7) and white matter RNA, annealed in PCR at 60°C (8).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–300

attempted (Materials and Methods, section 1.6.1), but RNA of suitable quality could not be isolated from these tissues (results not shown) while it could be done when frozen tissues were processed using this method. As a result, only frozen tissue specimens were included in the study. The guanidine isothiocyanate phenol-chloroform method of extraction was found to be less cumbersome than the pronase method, thus it was used on all the clinical specimens analyzed.

2. Reverse transcription-polymerase chain reaction

The RT-PCR technique was tested using different concentrations of reagents in order to achieve optimal amplifications without non-specific bands. The first reagent tested was $MgCl_2$, since the activity of *Taq* polymerase varies greatly with differing concentrations of $MgCl_2$. Following the determination of an optimal concentration of 4.0 mM $MgCl_2$ in the reverse transcription reaction and of 2.4 mM in the PCR reaction, the optimal concentration of dNTPs and quantity of primers were tested (Table 4). For the reverse transcription reaction dNTPs gave optimal results at 0.5mM and at 0.4 mM for the PCR. PCR primers were optimal when 50 pmoles each were added to the reverse transcription reaction and to the PCR (Fig. 8). Both primers were included in the reverse transcription reaction because negative stranded viral RNA is presumably present in cells infected with human coronaviruses

TABLE 4

OPTIMIZATION OF RT-PCR CONDITIONS

REVERSE TRANSCRIPTION

COMPONENT	AMOUNT OR CONCENTRATION TESTED		
dNTPs	0.25mM	0.5mM	**1.0mM**
primers	25 pmoles	**50 pmoles**	100 pmoles
$MgCl_2$ added	1.25 mM	**2.5 mM**	5.0 mM
*$MgCl_2$ (buffer)	1.5 mM	1.5 mM	1.5 mM
total $MgCl_2$	2.75 mM	**4.0 mM**	6.5 mM

POLYMERASE CHAIN REACTION

COMPONENT	AMOUNT OR CONCENTRATION TESTED		
dNTPs	0.0625 mM	0.125 mM	**0.25mM**
primers	25 pmoles	**50 pmoles**	100 pmoles
$MgCl_2$ added	0.5 mM	**0.9 mM**	1.9 mM
*$MgCl_2$ (buffer)	1.5 mM	1.5 mM	1.5 mM
total $MgCl_2$	2.0 mM	**2.4 mM**	3.4 mM

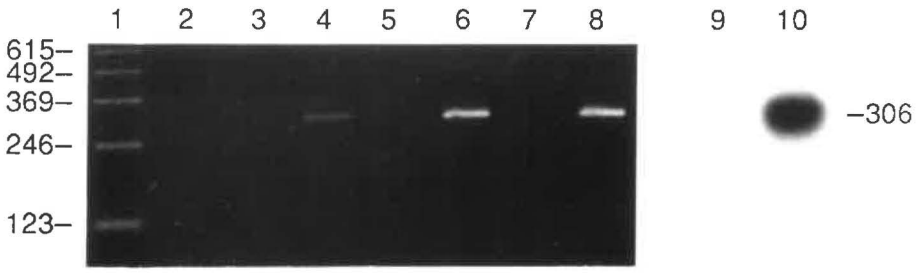
* 1X BIO/CAN *Taq* buffer contains 1.5 mM $MgCl_2$

Optimal amounts or concentrations utilized are marked in bold letters.

Figure 8: Evaluation of the effect of different amounts of primer on the quality and quantity of PCR amplification products.

Lane 1: 123 base pair DNA ladder.
Lanes 2-8: amplification products of RT-PCR using primer pair #1 for HCV-229E as visualized by ethidium bromide staining of agarose gels. Templates are amplified with increasing concentrations of primers: no RNA (2), 0.1 μg total RNA from mock-infected (3) and HCV-229E infected (4) L132 cells using 25 pmoles of primers in each of the reverse transcription and PCR reactions. 0.1 μg total RNA from mock-infected and HCV-229E infected cells in lane 5 and 6 respectively using 50 pmoles of primers. 0.1 μg of total RNA from mock-infected and HCV-229E infected L132 cells in lanes 7 and 8 respectively using 100 pmoles of primers.

Lanes 9 and 10: Autoradiography of hybridization of the product of lanes 5 and 6 with ^{32}P-labelled oligonucleotide probe specific for the target sequence on HCV-229E RNA, after transfer to nitrocellulose.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615–
492–
369–
246–
123–
–306

(Literature review, section 3), and thus the upstream primer could then be functional at this step. Amplification of human MBP RNA gave similar results in terms of the quantity of product at annealing temperatures of 55°C and 60°C (Fig. 7), thus 60°C was selected as the annealing temperature for all the PCR assays, since annealing at higher temperatures is presumably more specific. It was assumed that all primer pairs would behave in a similar fashion since they all had GC contents of 50 to 60%.

3. Estimation of the detectability level of the RT-PCR assay

In order to estimate the number of target sequences of RNA detectable using the assay, a quantifiable RNA template was prepared by cloning the HCV-229E amplification product of primer pair #1 in a pGEM®-3Z plasmid (Promega) and transcribing the target sequence *in vitro*, with incorporation of cytidine triphosphate labelled with ^{32}P. The amount of radioactivity incorporated is a function of the number of moles of radioactive CTP present in the transcript, thus the number of molecules of target RNA could be estimated.

3.1 Cloning of the HCV-229E amplification product

The amplification product of the first pair of primers for HCV-229E was cloned by blunt-ended ligation in the *Sma*I site of

the pGEM®-3Z plasmid multiple cloning site (Fig. 4). Five clones were obtained which gave positive signals when hybridized with the oligonucleotide probe for this pair of primers. The target sequence was analyzed for appropriate restriction enzyme sites which would yield fragments of predictable sizes depending on the direction in which the target sequence had been inserted (Fig. 9b). Mini-preps were made from three of the five clones and were digested simultaneously with *Eco*RI and *Pvu*II. The resulting fragments were visualized on an agarose gel seen in Fig. 9a. One clone in direct orientation and two clones in opposite orientation can be observed, although the molecular size markers 213, 234 and 267 do not align perfectly with the fragments observed. This could be due to differences in migration rates due to base composition.

3.2 *In vitro* Transcription

The plasmid was transcribed *in vitro* and the number of molecules present in 3µl of a 1/25 dilution of the transcription was calculated (Table 5) based on the comparison of the radioactivity incorporated in the transcription with that of an aliquot of [α-^{32}P]CTP. Subsequently, serial tenfold dilutions of this dilution were prepared and dilutions 10^{-1}, 10^{-2},10^{-3} and 10^{-5} were tested by RT-PCR (Fig. 10). At the lowest dilution, a hybridization signal was clearly visible and

TABLE 5

CALCULATION OF THE NUMBER OF TARGET SEQUENCES TRANSCRIBED

3 μl of a 15 μM solution of [α-^{32}P]CTP were counted with a scintillation counter giving 38 x 10^6 cpm.

$$\frac{38 \times 10^6 \text{ cpm}}{45 \times 10^{-12} \text{ MOLES } [\alpha\text{-}^{32}\text{P}]} = \frac{4.3 \times 10^5 \text{ cpm}}{\text{X MOLES } [\alpha\text{-}^{32}\text{P}]\text{CTP}}$$

$$\text{X} = \frac{45 \times 10^{-12} \text{ MOLES} \times 4.3 \times 10^5 \text{ cpm}}{38 \times 10^6 \text{ cpm}}$$

$$= 5.09 \times 10^{-13} \text{ MOLES } [\alpha\text{-}^{32}\text{P}]\text{CTP INCORPORATED}$$

$$5.09 \times 10^{-13} \text{ MOLES} \times 6 \times 10^{23} \text{ MOLECULES/MOLE}$$

$$= 3 \times 10^{11} \text{ MOLECULES } [\alpha\text{-}^{32}\text{P}]\text{CTP}$$

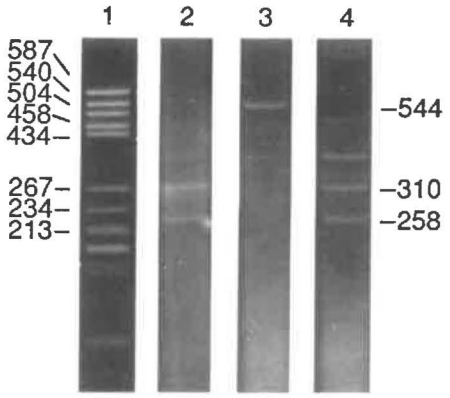
$$\frac{3 \times 10^{11} \text{ MOLECULES } [\alpha\text{-}^{32}\text{P}]\text{CTP}}{49 \text{ MOLECULES/TRANSCRIPT}} = 6 \times 10^9 \text{ target sequences transcribed}$$

Figures 9a and 9b: Restriction fragment analysis of recombinant plasmids and the predicted sizes of restriction fragments of directional and non-directional clones.

A: Visualiztion of restriction enzyme patterns on agarose gels stained with ethidium bromide. Lane 1: *Hae*III digest of pBR322 as a molecular size marker. Lanes 2 and 4: non-directional clones. Lane 3: directional clone.

B: Diagram of the predicted sizes of restriction fragments from clones in which the amplification product is inserted in direct or opposite orientation.

A


1
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587
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504
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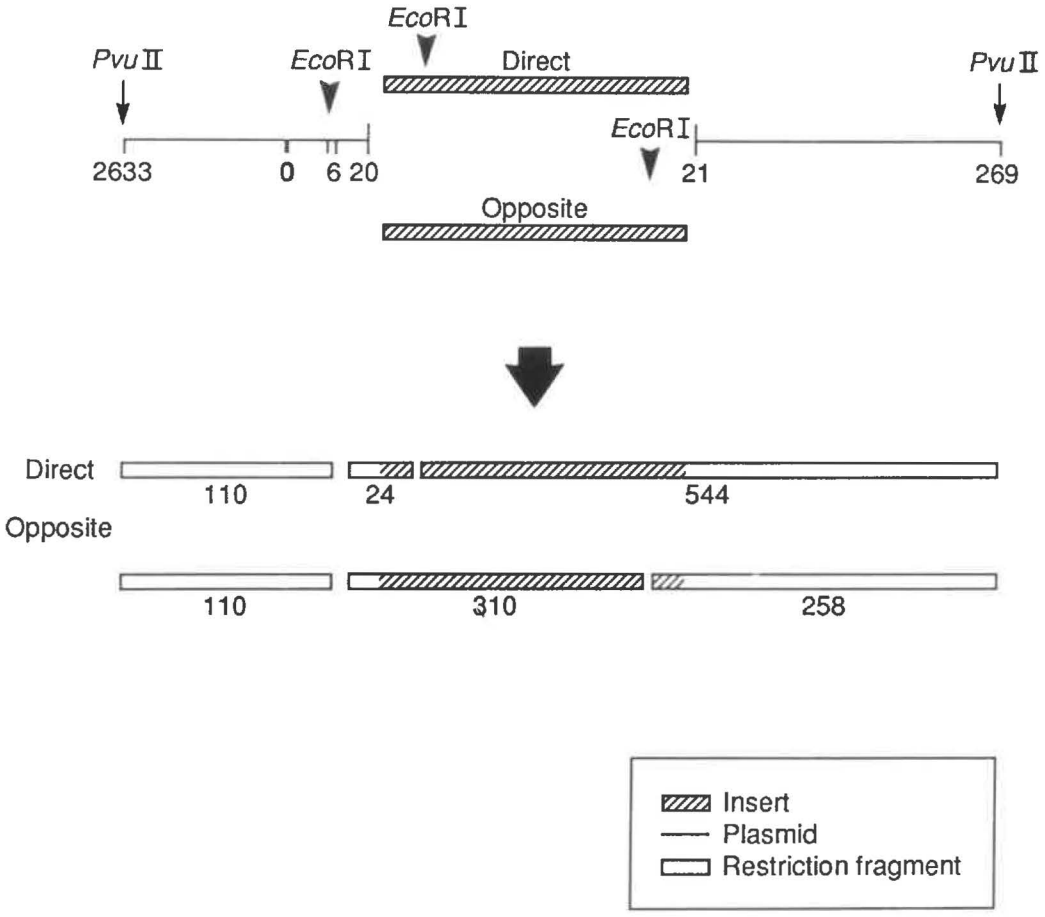
434
267
234
213
544
310
258

B


EcoRI
PvuII
EcoRI
Direct
PvuII
EcoRI
2633
0
6
20
21
269
Opposite
Direct
110
24
544
Opposite
110
310
258
Insert
Plasmid
Restriction fragment

a band was faintly visible on the agarose gel. It was calculated that 6 x 10^4 target sequences were present at this dilution.

4. Verification of the quality of RNA extracts

Frozen PBLs were tested for the presence of Γ-actin mRNA (Fig. 6) and were all found to be positive. Gray matter specimens were also tested for its presence and were all positive. White matter specimens were tested for the presence of myelin mRNA (Fig. 7), and six specimens were eliminated from the study due to the absence of detectable myelin mRNA. It was assumed that RNA degradation had occurred in these cases.

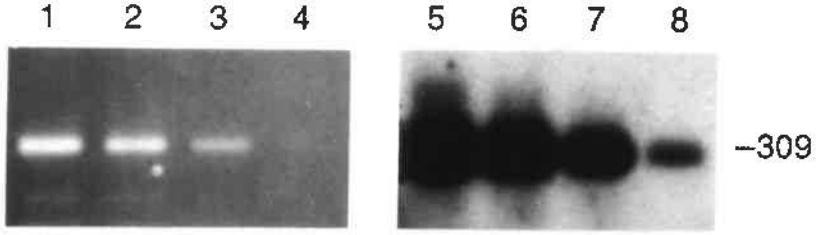
5. Analysis of clinical specimens for the presence of HCV-OC43 RNA

Thirteen specimens of PBLs from healthy individuals were analyzed for the presence of HCV-OC43 nucleic acid to verify whether PBLs could serve as a reservoir of persistent virus and although bands of the predicted size were visualized on agarose gels in five cases, no positive hybridization signal was obtained. Thirty two specimens of CNS tissue were analyzed, and again apparently non-specific bands could be seen in 14 of them, but no hybridization signal was obtained (Fig. 11).

Figure 10: Number of target molecules of RNA detectable with the RT-PCR assay for HCV-229E using primer pair #1.

Lanes 1-4: Visualization of amplification products on agarose gels by ethidium bromide staining. Amplification of 6 X 10^8, 6 X 10^7, 6 X 10^6 and 6 X 10^4 targets in lanes 1, 2, 3 and 4 respectively.

Lanes 5-8: Autoradiography of amplification products of lanes 1-4 with a ^{32}P-labelled oligonucleotide probe specific for the target sequence on HCV-229E, after transfer to nitrocellulose.


1
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8
–309

Figure 11: Non-specific amplification product in clinical specimens analyzed for HCV-OC43 RNA.

Lanes 1-7: Visualization on agarose gels of amplification products by ethidium bromide staining. Template in lanes 1-4 is total RNA extracted from 4 MS patients, from normal brain tissue in lane 5 and from HCV-OC43 positive control RNA in lane 6 and a 1/10 dilution of positive control RNA in lane 7.

Lanes 8-14: Autoradiography of hybridization of the amplification products in lanes 1-7 with a ^{32}P-labelled oligonucleotide probe specific for the target sequence on HCV-OC43, after transfer to nitrocellulose.

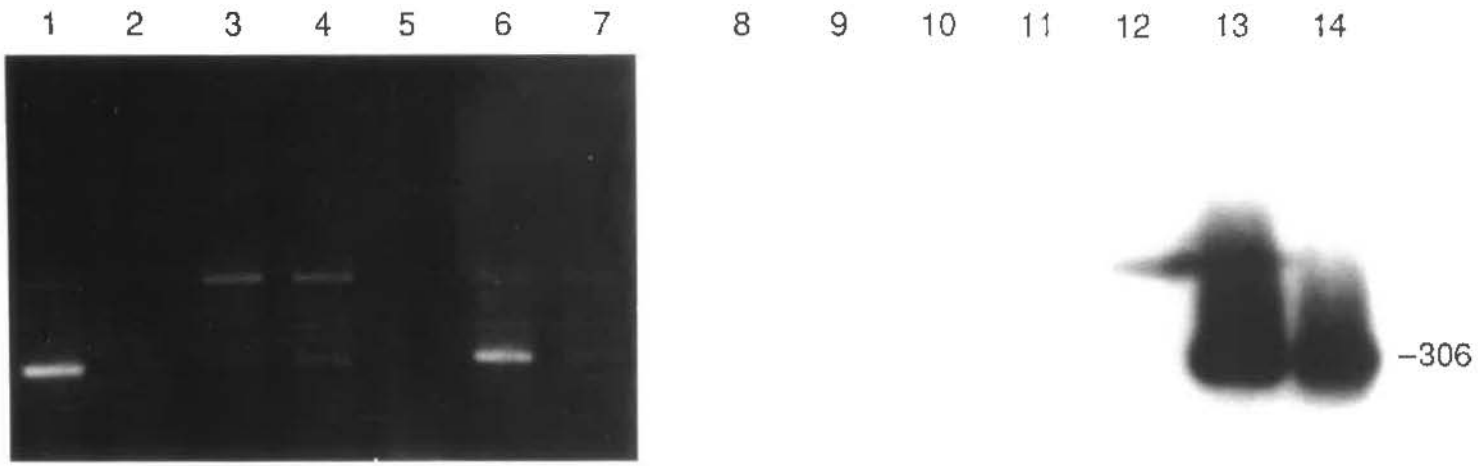

1 2 3 4 5 6 7 8 9 10 11 12 13 14
-306

TABLE 6
COMPARISON OF THE NUCLEIC ACID SEQUENCE OF THE NON-HYBRIDIZING AMPLIFICATION PRODUCT WITH HCV-OC43

AMPLIFICATION PRODUCT:	TTTACTGCTA AATCCACCTT CGACCTTCGA
HCV-OC43:	CAACCATCAG GAGGGAATGT TGTACCCTAC
AMPLIFICATION PRODUCT:	CCCTTAAGTT TCATAAGGGC TATCGTAGTT
HCV-OC43:	TATTCTTGGT TCTCTGGAAT TACTCAGTTT
AMPLIFICATION PRODUCT:	TTCTGGGGTA GAAAATGTAG CCCATTTCTT
HCV-OC43:	CAAAAGGAAA GGAGTTTGAG TTTGTAGAAG
AMPLIFICATION PRODUCT:	GCAACCTCAT GGGCTACACC TTGACCTAAC
HCV-OC43:	GACAAGGTGT GCCTATTGCA CCAGGAGTCC
AMPLIFICATION PRODUCT:	GTCTTTACCG TGGGTACTTG CGCTTACTTT
HCV-OC43:	CAGCTACTGA AGCTAAGGGG TACTGGTACA
AMPLIFICATION PRODUCT:	GTAGCCTTCA TCAGGGTTTG CTGAAGATGG
HCV-OC43:	GACACAACAG AGGTTCTTTT AAAACAGCCG
AMPLIFICATION PRODUCT:	CGGTATATAG GCTGAGCAAG AAGGTGGTGA
HCV-OC43:	ATGGCAACCA GCGTCAACTG CTGCCACGAT
AMPLIFICATION PRODUCT:	GGTTGATCGG GGTTTATCGA TTACAGAATA
HCV-OC43:	GGTATTTTTA CTATCTGGGA ACAGGACCGC
AMPLIFICATION PRODUCT:	GGCTCCTCTA GAGGGATATG A
HCV-0C43:	ATGCTAAAGA CCAGTAC

5.1 Cloning and sequencing of the non-hybridizing HCV-OC43 PCR product

To insure that the non-specific bands observed were not due to a virus related to HCV-OC43, one of the amplification products was re-amplified, cloned and sequenced. The sequence of the non-hybridizing band and the corresponding sequence of HCV-OC43 RNA can be seen in Table 6. No significant degree of nucleic acid identity was observed in any region of the amplification products. Amino acids were also compared based on the predicted amino acid sequences from three different reading frames on the sequence of the non-hybridizing product (Table 7). On the basis of these results we can safely state that the specimens were negative for HCV-OC43 as no hybridization signal was observed in any of them (Table 8). The GenBank™, version 70 (January, 1992) nucleotide sequence database, was searched for related nucleotide sequences and the highest degree of homology (181/254 nucleotides) was observed between this sequence and human mitochondrial tRNA genes (Anderson *et al.*, 1981), indicating that a cellular RNA was non-specifically amplified in some instances.

6. Analysis of clinical specimens for the presence of HCV-229E RNA

Thirteen specimens of PBLs from healthy individuals were tested for the presence of HCV-229E nucleic acid and six of

TABLE 7

ABSENCE OF AMINO ACID IDENTITY OF THE HCV-OC43 NON-HYBRIDIZING BAND WITH THE TARGET SEQUENCE IN THREE READING FRAMES

READING FRAME	IDENTITY
1	7%
2	0%
3	9%

TABLE 8

ANALYSIS OF CLINICAL SPECIMENS FOR HCV-OC43 RNA

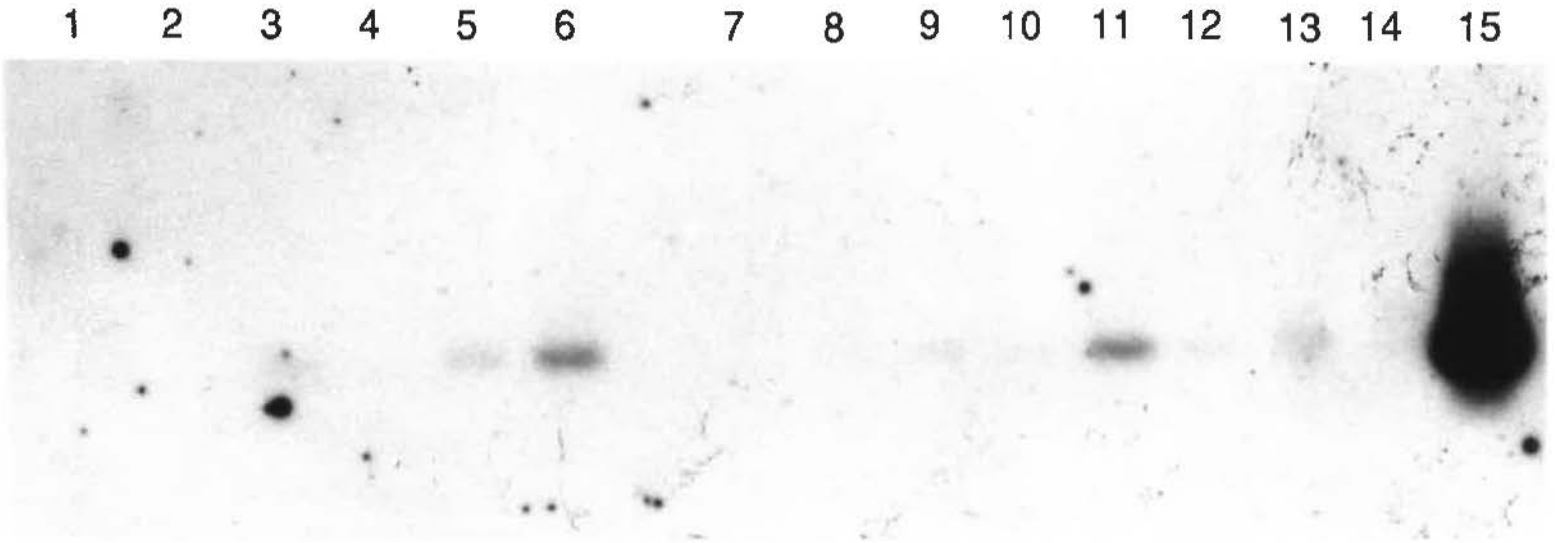
SPECIMEN	POSITIVE	NEGATIVE	TOTAL
PBLs	0	13	13
CNS TISSUE	0	32	32

these gave positive hybridization signals (Fig. 12). Upon re-testing either the same extraction or a new extraction of the same PBLs, the specimens from three of the original six individuals gave positive results a second time. Two initially negative specimens were positive when re-extracted and re-tested. These results imply a limited reproducibility of the test. However, they also suggest that some individuals harbor HCV-229E RNA in PBLs.

While carryover contamination involving entire RT-PCR runs was not occurring, spot contaminations of unknown origin were occurring and therefore it was considered necessary to analyze specimens with a second set of primers. HCV-229E primer pair #2 was synthesized, along with a corresponding internal oligonucleotide to serve as a probe. The new primers gave weaker signals when tested in parallel with HCV-229E primer pair #1. Thus it was possible to confirm positive specimens with a second pair of primers in some, but not in all cases. It was also considered necessary to include negative controls for RNA extraction as well as RT-PCR. Mouse brain tissue was selected as a negative control tissue on the assumption that human coronavirus would be absent in murine tissue. Two specimens of mouse brain were extracted simultaneously with every eight specimens of human CNS tissue and tested by RT-PCR at the same time.

Figure 12: Detection of HCV-229E RNA in extracts of PBLs from healthy adults with primer pair #1.

Autoradiography of amplification products migrated on agarose gels, transferred to nitrocellulose and hybridized with a ^{32}P-labelled oligonucleotide probe specific for the target sequence of HCV-229E RNA. Lanes 1-13: Template is total RNA extracted from 10^6 PBLs each of 13 healthy adults, no RNA (lane 14) and positive control RNA (lane 15).


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Another phenomenon which became apparent as the study progressed was that, for unknown reasons, some RT-PCR runs detected lower concentrations of target than others. To address this problem, two positive controls were included in subsequent tests: 160 pg of total RNA from infected cells and a weak positive control of 1.6 pg of the same RNA. The weak positive control gave a signal of a low intensity, comparable to those obtained in the clinical specimens, which were only visible after hybridization and were not visible on the agarose gels stained with ethidium bromide.

Twenty eight specimens of CNS tissues, 17 MS and seven controls, were extracted twice and each RNA extract was analyzed at least once for the presence of HCV-229E RNA. An additional four specimens of MS tissue were extracted once only but aliquots of the same extraction were tested at least twice. There was still evidence of spot contamination in the mouse brain control RNA on some runs which led us only to count as positive those specimens which gave positive results on two separate RT-PCR tests in which the negative controls did not show evidence of contamination. These were termed confirmed positives. Similarly, specimens which produced negative results on every test were considered negative. Four specimens of tissue from MS patients were confirmed positive while eight were negative. Three of the four confirmed positive specimens

TABLE 9

ANALYSIS OF CNS TISSUES FOR HCV-229E RNA

SOURCE	POSITIVE	NEGATIVE
MS	4	8*
NORMAL	0	1
ALZHEIMER'S	0	4
MENINGO-ENCEPHALITIS	0	1
ISCHEMIC VASCULAR DISEASE	0	1

* from 6 patients (all other numbers also correspond to the number of patients tested).

were tested with two different sets of primers and were positive with both of them. Seven specimens of control tissue were negative, one from normal, four from Alzheimer's tissues and two from patients with other neurological diseases were all negative (Table 9).

All of the confirmed positives occurred in CNS tissues from MS patients. Positive results occurred in specimens of grey matter and normal appearing white matter as well as of plaque tissue (Table 10).

Other tissues gave less clearcut results due to evidence of contamination in controls, or else because they were initially positive but this result could not be confirmed on subsequent assays. For the purposes of tabulating the results from all the specimens, the remaining specimens were divided into two groups. Specimens that were initially positive, and were positive in a second test in which evidence of possible contamination in the negative controls was observed were termed unconfirmed positives. Initially positive specimens which were negative upon re-testing were termed indeterminate. The results obtained for each CNS tissue specimen are presented in Table 10. Typical results are presented in Figure 13.

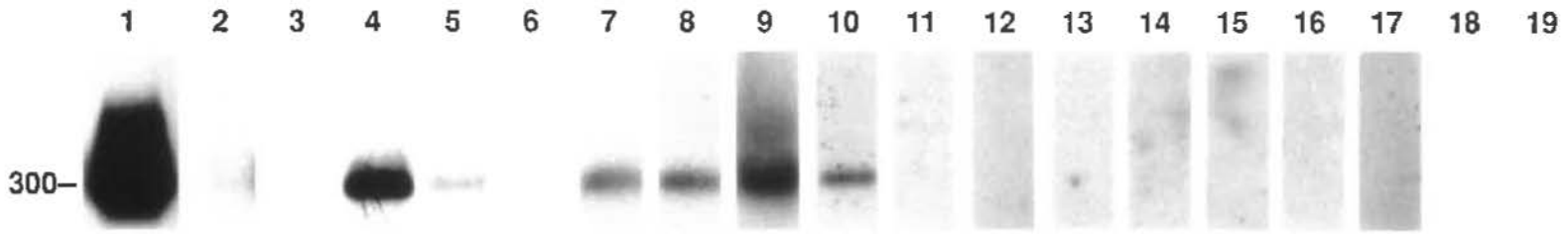
TABLE 10
RESULTS OF RT-PCR DETECTION OF HCV-229E IN CNS TISSUES

PATIENT		SOURCE OF TISSUE	RESULT
MS	1	plaque	unconfirmed positive
		white	indeterminate
	2	white*	-
		plaque	unconfirmed positive
		white	unconfirmed positive
	3	plaque	-
		white	unconfirmed positive
	4	plaque	-
		plaque	+
	5	plaque	indeterminate
		plaque	-
	6	white*	-
		plaque*	-
		white*	-
		white	+
		plaque	unconfirmed positive
	7	white	+
	8	grey	unconfirmed positive
	9	grey	+
	10	plaque	unconfirmed positive
	11	grey	-
NORMAL	1		-
	2		indeterminate
	3		indeterminate
	4		indeterminate
	5		-
ALZHEIMER'S	1		-
	2		-
	3		-
	4		-
MENINGO-ENCEPHALITIS			-
ISCHEMIC VASCULAR DISEASE			-

white: normal-appearing white matter, plaque: plaque tissue
grey: grey matter. * one RNA extraction tested

Figure 13: Detection of HCV-229E in RNA extracts from CNS tissues.

Hybridization of amplification products with a ^{32}P-labelled oligonucleotide probe specific for the target sequence on HCV-229E. The templates in each lane are RNA extracted from: Lane 1, L132 cells infected with HCV-229E; lane 2, mock-infected L132 cells; lane 3, no RNA; lane 4, mouse brain mixed with 160 pg HCV-229E RNA; lane 5, mouse brain mixed with 1.6 pg HCV-229E RNA; lane 6, mouse brain; lanes 7-14, CNS tissues from MS patients (lanes 7 and 9, white matter; lane 8, grey matter; lane 10, plaque tissue); lanes 15-19, specimens from control brains (lane 15, normal; lanes 16 and 18, Alzheimer's; lane 17, ischemic vascular disease; lane 19, subacute meningo-encephalitis).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DISCUSSION

The RT-PCR technique for detecting HCV-229E and HCV-OC43 RNAs was set up and a pilot study of multiple sclerosis tissues for the presence of the genomes of these two viruses was carried out. The first observation made during the course of the study was that formalin-fixed tissues are not suitable specimens for the extraction of RNA. Indeed, it is becoming clear in the literature that paraffin-embedded, formalin-fixed tissues are sometimes suitable for RNA extraction and amplification but that formalin fixation is detrimental to RNA (Godec *et al.*, 1990). This group examined cases of subacute sclerosing panencephalitis for the presence of Measles virus RNA in paraffin-embedded, formalin-fixed CNS tissues. Success was due to the fact that virus is highly abundant in such tissues, and also due to the large size of the blocks. The authors hypothesized that formalin does not fully penetrate large blocks before it is removed by paraffin embedding. Recent studies of tissue fixation indicated that ethanol fixation of tissues prior to paraffin embedding is preferable if RNA is to be analyzed and that formalin fixation is virtually useless (Ben-Ezra *et al.*, 1991).

After failing to obtain RNA of sufficient quality from the formalin-fixed tissues available to us, only frozen tissues were considered for inclusion in the study. The guanidine isothiocyanate-phenol-chloroform method of RNA extraction was

selected as the method of choice because it involved fewer manipulations than the pronase method. The pronase method would have required an additional DNA digestion step whereas the method selected had already given good yields of pure RNA. No DNA coding for Γ-actin or MBP was ever detected by PCR in all of the specimens studied.

The RT-PCR strategy adopted in the study appears to have been successful, all five primer pairs, HCV-229E primer pairs #1 and #2, and the pairs for HCV-OC43, Γ-actin and MBP proved to be specific for their respective targets, when confirmation by hybridization was employed. The analysis of specimens for MBP or Γ-actin mRNA made it possible to eliminate those of inferior quality. MBP mRNA appeared to be less abundant than Γ-actin RNA is in white matter, an observation based on the fact that MBP mRNA negative specimens would at times remain positive for Γ-actin RNA but not vice versa. It is also possible that the primer pair for Γ-actin mRNA amplified its respective target sequence more efficiently than did the MBP primer pair. Extensive comparisons of the detectability levels of each set of primers was beyond the scope of this project. Nevertheless, the use of these primers as indicators of RNA quality was a simple and effective means of evaluating archival tissues for the degree of preservation of RNA. It might be worthwhile to further characterize these primer pairs in terms of the number

of molecules they detect for future studies of RNA viruses in archival tissues.

The level of detectability of the RT-PCR assay for HCV-229E RNA as assessed by the *in vitro* transcription of target molecules gave a lower limit of 60,000 molecules detectable by this primer pair. This is certainly an overestimation of the number of molecules present because incomplete transcripts would inflate the calculation of the number of molecules of [α-^{32}P]CTP incorporated and also because the radioactive molecules incorporated in the template were causing continual damage to it through radiolysis. Nevertheless, it gives a rough idea of the number of molecules present. It would have been preferable to test a pure preparation of genomic viral RNA, but since unknown molar quantities of mRNAs are associated with virions (Abraham *et al.*, 1990), it was considered to be a less accurate method of estimation.

While this is a high value when compared with the low numbers of copies of DNA detectable using PCR (Sninsky and Kwok, 1990), it compares quite favorably with the estimates of copies of RNA amplifiable using RT-PCR to detect other RNA viruses. A similar signal is obtained with amplification of 10^{-7} pmoles, or roughly 60,000 molecules of an RNA transcript of cloned HIV-I provirus (Murakawa *et al.*, 1988). Interestingly, 1.6 pg of total RNA from HCV-229E infected cells gives a

similar hybridization signal after amplification as 10 ng of total RNA from HIV-I-infected H9 cells did in Murakawa's study. Likewise, total RNA from influenza virus H1-infected cells was detectable when a minimum of 20 cells were tested (Bressoud *et al.*, 1990). In our RT-PCR, positive signals were obtained when the total RNA (1.6 pg) from the equivalent of 0.1 cells was tested. In another RT-PCR, the authors demonstrated that the RNA from $10^{2.5}$ (316) $TCID_{50}$ of rhinovirus in nasal washings from infected volunteers is easily detected (Gama *et al.*, 1989). The number of molecules of viral RNA present in addition to RNA incorporated into infectious virions is impossible to estimate, however it is likely to be far more abundant, bringing the level of detectability of that RT-PCR within the same range as the technique used in the present study.

Evidently, it appears to be the RNA reverse transcription step that reduces the overall level of detectability, given the results with the other RT-PCRs which have been characterized as described above. Secondary structures in RNA may be responsible for a lower reverse transcription efficiency. It would therefore be advisable to optimize the efficiency of the reverse transcription step separately to lower further the level of detectability of the RT-PCR technique.

Thus far, the ability of primer pairs to amplify DNA efficiently depends on the target sequence, which is defined by

the primer pairs. All primer pairs employed in this study had GC contents of 50 to 60% (Saiki, 1989), as recommended in the literature, and all were roughly 300 bases in length, which is a relatively small size that should place no constraint on amplification. It is unlikely that the size or GC content we employed brings to bear on this question, since we observed varying levels of detectability from one primer pair to another, the primer pair for Γ-actin appearing to be a more powerful amplifier than the pair for MBP. Thus far, the level of detectability of different primer pairs in PCR tests on DNA is determined by empirically testing them in PCR reactions.

While we were surprised to find that the PCR test required a large number of molecules of RNA to be present to give a positive result, this was not a serious drawback in the study since it was RNA that was being detected. The target sequence is located on the viral mRNA coding for the nucleoprotein, which is by far the most abundant viral mRNA in infected cells. Furthermore, this sequence is also present on all the other six viral mRNAs including the genome (Fig. 2), probably more than doubling the number of target sequences present. Therefore, we estimate that a single infected cell would harbor a similar quantity of this target sequence and should thus be detectable using this assay.

The reproducibility of the assay is another issue to consider. The RT-PCR assay was specific and consistently negative in the testing of HCV-OC43. However, due to the obtention of positive results with the HCV-229E assay and the need to confirm these results in subsequent tests, more experience was acquired with it. Many factors influence reproducibility with the reverse transcription-polymerase chain reaction. One important factor is RNA stability. We were at the lower limit of detectability using the assay, and while RNA samples were aliquoted before freezing, the best results were nevertheless obtained when RNAs were tested immediately after extraction without an intervening period of storage at -70°C. Another factor appears to be the stability of the enzymes, reverse transcriptase or possibly *Taq* polymerase, in spite of its heat stability. Amplification of the weak positive control varied in efficiency from one assay to the next regardless of whether the same reagent preparation was employed.

Finally, there was possible contamination with template, whether it be RNA, cDNA or amplicons. It would be very useful to be able to identify the source of contamination, and while it is impossible to prove beyond doubt what the source is, a likely candidate can be singled out by a process of elimination. It does not appear to be carry-over contamination with amplification products (amplicons) which is the typical

source of contamination in PCR assays. Interestingly, no contamination was experienced in testing HCV-OC43, which would be expected if carry-over contamination was occurring. It is unlikely that the contamination was due to RNA, because, as we have already seen, a large number of molecules would be required to produce a signal and this quantity would not be present in aerosols arising from the specimens. Furthermore, RNA would not be very stable on work or equipment surfaces and would certainly not be present in sufficient quantities to produce contamination. Positive control RNA was always processed last when setting up RT-PCR assays, and did not constitute a problem with HCV-OC43, therefore there is no reason to suppose that HCV-229E positive control RNAs would be a source of contamination. Furthermore, specimen RNAs were extracted separately from positive control RNA, and, during the second half of the study, they were air-dried to avoid risk of contamination by viral RNA or virally derived DNA in the SpeedVac Concentrator. We have no indication as to the efficiency of amplification of the target sequence on DNA as opposed to RNA, but the contamination we witnessed must arise from DNA if it cannot be attributed to RNA, and therefore DNA is probably amplified more efficiently.

Another frequently cited source of contamination in PCR is clones containing the target sequence being amplified (Mahony,

1990). In fact, such a clone of HCV-229E had been employed in the coronavirus laboratory two years previous to the present study. It is very unlikely that this clone could be causing contamination two years later in a different laboratory from the one in which it was produced. Furthermore, HCV-OC43 was also being both grown and cloned in the virus laboratory, and while it is not the N-gene that was cloned, no problem of contamination or potentially false positives was ever experienced in testing for its RNA.

That leaves us with the last candidate contamination template: cDNA. If, indeed, DNA is more readily amplified than RNA, then there is certainly a risk involved at the additional step of RT-PCR when the cDNA produced through reverse transcription is transferred to the PCR reaction in a separate tube, and this manipulation is repeated for all the specimens in that RT-PCR run (ten in our case). The positive controls were always transferred last and therefore presented no risk, but aerosols containing cDNA could potentially have been produced when the reverse transcription reaction tubes of positive specimens were opened. Thus it appears that the most likely source of contamination is cDNA from positive specimens. In future studies it would be worthwhile to employ a protocol that does not involve the additional step of transferring the cDNA to the PCR reaction tube, such as the one used by Godec *et*

al. (1990), in the study of SSPE CNS tissue. The disadvantage in using such a protocol in which the reverse transcription and PCR reagents are all placed at once in the same volume in the same tube is that more molecules of RNA must be present in order to obtain a signal (Godec, personal communication).

The RT-PCR tests for HCV-OC43 RNA reveal the importance of confirming the nature of amplification products by some method, either restriction enzyme digestion or hybridization or sequencing. We proceeded with sequencing in this particular case because of the high degree of nucleotide homology shared between antigenically related coronaviruses infecting the same species, as in the case of MHV or even between coronaviruses infecting different species, such as between bovine coronavirus and HCV-OC43 (Kamahora *et al*., 1989). By sequencing and analyzing the non-hybridizing amplification product, we ruled out the possibility that we were detecting a strain of coronavirus related to HCV-OC43. The detection of coronavirus genomes using a probe derived from MHV-A59 and a probe from the MS isolate in multiple sclerosis tissues (Murray *et al*., 1992b) by *in situ* hybridization further demonstrates the need for eliminating this possibility. To eliminate it completely, it would be advisable to synthesize an oligonucleotide probe specific for the non-hybridizing product and determine whether it is the same nucleic acid detected in all specimens, PBLs as

well as CNS tissues, since it was observed in some, but not all PBL RNAs studied. That, however, goes beyond the objective of this study which was to analyze specimens for HCV-229E and HCV-OC43 RNAs. On the basis of the results obtained in CNS tissues, we can safely say that HCV-OC43 is absent in the samples studied, thus confirming the results obtained by Sorensen *et al.*, 1986, in four MS brains using dot blot hybridization. We did not detect HCV-OC43 RNA in the 13 specimens of PBLs analyzed, indicating that this virus is not usually present in these cells in healthy adults at the levels we were detecting. The sample is too small to draw any final conclusions, but the results strongly suggest that this virus does not infect PBLs in healthy adults.

In PBLs, three of thirteen healthy adults consistently gave positive results for HCV-229E RNA. In a study of this small size, it was considered of no interest to continue examining PBLs of MS patients for human coronavirus genomes in view of the positive results obtained with healthy adults. It is nevertheless a highly interesting result in that infection of PBLs by HCVs has not previously been documented, although infection of macrophages by MHV strains *in vitro* is well-documented and is mouse strain dependent (Bang and Warwick, 1960). Moreover, MHV-3 is known to replicate in T and B lymphocytes *in vitro* as well as in macrophages (Lamontagne *et*

al., 1989). This opens up an area of research into the factors influencing HCV infection of PBLs such as the type of cell infected, the disease status of the individual harboring HCV genomes in PBLs, the time frame of PBL infection and possibly genetic factors controlling susceptibility of PBLs to infection. There is also the possibility of the spread of HCVs to other organs, be they the CNS or other. In the animal models, susceptibility to hepatitis correlates well with susceptibility of macrophages to infection *in vitro*. PBLs are certainly a potential vehicle for HCV-229E to reach the CNS, or, as is discussed below, they may be responsible for the signals detected in CNS tissues.

Interestingly, the cellular receptor for HCV-229E has recently been identified as aminopeptidase N (Yeager *et al.*, 1992) which is identical to human myeloid plasma membrane glycoprotein CD13 (Delmas *et al.*, 1992). It is expressed on a variety of different cells, notably macrophages, granulocytes and on synaptic membranes prepared from cells of the central nervous system (Look *et al.*, 1989). The presence of the cellular receptor for HCV-229E on these cells suggests a possible susceptibility of these cells to infection, which further supports our evidence of the presence of viral RNA in PBLs as discussed above, and in the central nervous system.

We were able to confirm the positive results obtained for HCV-229E in four of 11 MS brains. The positive results obtained in CNS tissues raise a series of issues. First would again be the type of cell infected. It must be borne in mind that the RNA tested is extracted from a tissue homogenate which contains blood vessels as well as the neural and glial cells. It may well be that the HCV RNA detected in this study arose from PBLs in the brain vasculature, given the low number of molecules detected. However, signals from CNS tissues were generally stronger than those in PBLs indicating that the viral nucleic acid was present in higher concentrations in the CNS tissues than in the PBLs examined. Study of positive specimens by *in situ* hybridization is in order to identify the cell type(s) harboring the viral genome. Due to stronger and more frequent positive signals for HCV-229E obtained in CNS tissues, the results suggest that this is a neurotropic virus under certain, as of yet undetermined, conditions. Once the cells containing the viral genome are identified, methods to verify viral gene expression would aid in characterizing the type of infection to which these cells are subjected. As discussed in the bibliographical review, evidence for gene expression of HCV-229E in cultured neuroblastoma cells as well as in a cell line derived from an oligodendrocyte-rhabdomyosarcoma fusion indicates that infection of neurons and perhaps oligodendrocytes by this virus is indeed possible and further

in vitro studies of the kinetics of infection in these cells will provide additional insight into the pathogenic potential of the virus in this tissue.

A second issue is how many tissue specimens were positive. This is a question that may only be answered using a more refined technique. Because of the problems of possible contamination, most likely by cDNAs of positive samples, this RT-PCR for HCV-229E allows us to confirm that HCV-229E RNA is present in some CNS tissue, more particularly that positives were confirmed in four specimens, but we cannot answer for the status of an additional seven which appeared to be positive. Since this is a pilot study involving a small number of specimens, a quantitative statistical analysis was not appropriate in any case. Fewer control tissues were available than MS specimens which would make any quantitative analysis attempting to establish a disease association completely biased. Our positive findings now justify conducting a more rigorous study of HCV genomes in the CNS, requiring either the employment of a more precise technique or the development of a system with more reliable negative controls.

It is worthy of note that not all the specimens from the same brain gave similar results and also that grey matter as well as plaque tissue and white matter gave positive results. It would not be surprising that virus would not be evenly

distributed in the tissues. If the virus is transported by or is present in PBLs, an uneven distribution of virus in brain tissue is to be expected. If the virus is responsible for producing sclerotic plaques by direct infection of glial cells, it is known that plaques are distributed in MS brains in what appears to be a non-systematic fashion. Even if the virus also travels to the brain along the olfactory and trigeminal nerves, as does MHV when inoculated intranasally (in fact the natural route of entry of MHV) in one model of chronic demyelination, an uneven distribution in CNS tissues would be observed as virus spreads along contiguous nerve tracts (Perlman *et al.*, 1990). Studies on the route of entry to the CNS of poliovirus indicate that it may reach the brain both via nerve tracts and through the bloodstream (Morrison and Fields, 1991). For the purposes of future studies, if neurotropism is confirmed, these observations underline the importance of collecting more than three specimens per patient, preferably in different areas of the brain including grey matter. Furthermore, the same collection protocol would necessarily have to be applied to the control patients. Unfortunately, it is very difficult to obtain frozen CNS tissues collected according to a standard protocol. Due to their scarcity, they are available from different sources, if it all, in locations across the country and therefore they are sent from a variety of facilities, such as banks or laboratories. It is hoped that with widespread

diffusion of these results that more tissues will be made available for study, particularly tissues from patients of inflammatory neurological diseases.

The final observation to be made on the analysis of CNS tissues is that the presence of HCV-229E nucleic acid was only confirmed in specimens from MS patients. Since this was a pilot study, and not a controlled, statistical study, no conclusions as to the implication of the virus in the etiology or the pathogenesis of MS can be made at present. PCR and RT-PCR are in their infancy, and as such many tissues, but particularly CNS tissues, have not been characterized in terms of the detection of low concentrations of viral nucleic acid and the significance of their presence. Furthermore, the low number of molecules detectable by this technique brings with it the disadvantage of contamination of specimens with exogenous DNA, thus to date, it is not a method which is readily applicable to rigorous, quantitative studies. Therefore the results obtained using this technique must be interpreted with caution. In this study, lessons learned in previous PCR studies were taken into account both regarding the control of carryover contamination (Kwok and Higuchi, 1989) and the need for confirming positive specimens in subsequent assays. The initial reports of positive results for HTLV-I DNA in the PBLs of MS patients proved not to

be reproducible in later, more rigorous studies (Erlich *et al.*, 1991).

In the present study, four samples were confirmed positive after re-testing. Before considering the fact that they arose from four different MS brains, it is important to note that this human coronavirus is likely neurotropic, based on this study, on the basis of *in vitro* studies and on the fact that the cellular receptor for the virus is present on cells of the CNS. Regardless of the circumstances permitting its presence in the brain, the question remains as to whether it is pathogenic in the state in which it is found in the CNS. Obviously, the presence of viral genomes does not automatically imply that the virus is causing damage to the tissue, but it is certainly a possibility worthy of further investigation. Additionally, HCV-229E RNA may be detectable in the CNS of patients with other neurological diseases as well as in MS or in normal brains when more extensively sampled, or both. In the former case, HCV-229E may exacerbate those conditions as an agent of secondary infection in tissues undergoing injury from other causes.

However the results are not inconsistent with the hypothesis that HCV-229E is an etiologic agent of MS. These results constitute additional evidence in support of such an hypothesis. The observation of coronavirus-like particles by Tanaka *et al.*, 1976, in one MS brain was the first direct

evidence that a coronavirus could be responsible for causing MS. Other lines of evidence also indirectly support the implication of a human coronavirus as an etiologic agent of MS. The existence of the murine coronaviral models of demyelinating disorders suggests that a similar pathology could be caused by a human coronavirus as does the *in vitro* infection of neuroblastoma cells.

Epidemiologic studies of the occurence of MS in migrant populations indicate that the incidence in MS in populations which migrated from a region of high risk for MS to a region of low risk for MS after, but not before, the age of 15 will result in the same elevated incidence of MS associated with the high risk region. This would imply that it is exposure, or the lack of it, to an environmental agent before the age of 15 which at least partially determines the risk for MS. Scant information is available about the likely age of first infection with HCV-229E, however in two studies, antibody to HCV-OC43 is detectable in very young children while antibody to HCV-229E appears later (M^{c}Intosh *et al.*, 1970). Differences in the absolute proportion of antibody positive individuals and in their age distribution varied in different regions of the same country (Hruskova *et al.*, 1990). Generally speaking, the percentage of antibody-positive individuals increases with age, reaching values of 70 to 90%. Studies of the age distribution

of exposure to HCV-229E in areas of low and high risk for MS may in the future reveal an epidemiological link of this virus with MS. Another point of interest is the amino acid identity shared between HCV-229E and human MBP and which partly coincides with an immunogenic portion of human MBP (Jouvenne *et al.*, 1992). This suggests molecular mimicry as a possible mechanism provoking an autoimmune response if indeed HCV-229E proves to be implicated in the etiology of MS.

The observation of coronavirus-like particles by electron microscopy and the murine model of demyelinating disorders are likewise not inconsistent with the possibility that Burks' isolate of coronavirus from two MS patients may be the causative agent of MS. Initially, the report of a greater than 90% nucleotide identity between his isolate and that of MHV-A59 made it appear very likely that a laboratory contamination was responsible for his results. However, recent reports of very high percentages of nucleotide homologies shared between coronaviruses causing diseases in entirely different species as mentioned previously makes his isolation from CNS tissues appear more plausible than it did in the past. A probe derived from the SD isolate was used to detect homologous sequences in 11 of 21 MS brains by *in situ* hybridization and in two of 21 controls. In addition, the same isolate was used to inoculate an owl monkey intracerebrally which produced demyelination

(Murray *et al.*, 1992b) indicating that this virus is capable of infecting primates and of producing pathological changes reminiscent of MS in them.

The same group has further extended studies by *in situ* hybridization by analyzing tissue sections adjacent to the ones which were originally positive and testing for additional viruses, and they continued to find sequences homologous to the SD isolate in 12 of 22 MS brains (Murray *et al.*, 1992a) Interestingly, they were able to detect antigen using a polyclonal anti-MHV-A59 antiserum as well as monoclonal antibody to MHV-JHM in sections from two of 22 MS brains. They found no evidence for the presence of HCV-229E genomes. This could be explained by the fact that in positive brains, the tissue sections were selected for harboring SD/MHV-A59 or possibly because the RT-PCR technique detects fewer molecules. Unfortunately, no positive control for HCVV-229E appears to have been employed in this study making it impossible to arrive at a firm conclusion regarding the absence of the nucleic acid of this virus. At present, there is no additional evidence that the SD coronavirus isolate infects humans, there is no information concerning a possible route of infection, and even less information is available about the epidemiology of human infection by such a virus. It is worthy of note that HECV, which has not yet been sequenced, is antigenically

related to HCV-OC43 of antigen group II, to which MHV-A59 also belongs, and perhaps the enteric rather than the respiratory route of infection is employed by the SD isolate in humans.

It is possible that more than one infectious agent causes MS, although epidemiologic findings suggest otherwise. The occurence of cases of MS on the Faroe Islands for the first time in 1943 suggests that a single agent was introduced in the preceding years. If it were an infectious agent such as a virus, Kurtzke and Hyllested (1986) hypothesize that it is a ubiquitous agent transmitted to a large portion of the population, but causing MS only in susceptible individuals. This is again consistent with a virus such as HCV-229E, which causes the common cold. However, the existence of geographic regions of low to high risk may reflect the circulation of different viruses in the various regions.

Therefore the results obtained are not inconsistent with a hypothetical role for HCV-229E in the etiology of MS and other lines of evidence also support this possibility. Infection of the CNS by more than one virus could occur as a result of MS, and of perturbations of the blood-brain barrier which sometimes accompany this disease. Thus, it is premature at present to single out HCV-229E over candidate viruses such as Burk's isolates, the retroviruses detected by Perron *et al.* (1991) in monocyte cultures from MS patients, or others whose

presence in MS tissues may be revealed in future PCR studies.

While it is beyond the scope of this study to resolve the many questions raised by the results found, it should be possible in the future to characterize the infection of PBLs by HCV-229E, to verify its *in vivo* neurotropism and eventually to clarify its possible role in neurologic disease.

CONCLUSION

The first objective of the study was to set up the RT-PCR technique for the analysis of tissue specimens for the presence of HCV nucleic acids. An operative system was created involving a micro-method for the isolation of RNA, an RT-PCR assay for testing the quality of RNA isolated from archival CNS tissue and finally the technique for detecting viral RNA with a demonstrated capacity to amplify nucleic acid sequences in clinical specimens.

The second objective of the project was to conduct a pilot study of MS and control tissues for the presence of HCV RNA. The analysis of PBLs and CNS tissues for HCV-OC43 nucleic acid gave negative results in 32 CNS specimens from 22 individuals and in 13 specimens of PBLs from healthy adults, leading us to conclude that HCV-OC43 is not normally present in the PBLs or CNS of adults at the concentrations we could detect. In contrast, evidence for HCV-229E genome expression was found in both types of sample, although the results in PBLs are preliminary due to problems with reproducibility. The suggested presence of HCV-229E nucleic acid in the PBLs of healthy adults is an extremely interesting result since a viremic state during or following infection has not previously been documented for this virus and a variety of studies to confirm and to characterize HCV-229E infection of PBLs is in order.

The four positive specimens of CNS tissue came from MS patients and not from controls. Since PCR is performed on extracts of tissue homogenates, other techniques which permit the identification of the cell type harboring the viral products should be implemented to confirm the neurotropism of HCV-229E suggested by these results, especially in view of our own results in PBLs. While this is not a statistically controlled, quantitative study, the positive results obtained in CNS tissue from MS patients are certainly not inconsistent with a coronaviral etiology of MS. On the contrary, the results of this study warrant a larger study including controls from other inflammatory neurological diseases which would further clarify the possible implication of HCV-229E as etiologic agent or as an agent of secondary infection during the course of such diseases.

ACKNOWLEDGMENTS

I would like to thank Mr. Claude Pichette, the director of the Institut Armand-Frappier, for providing the facilities necessary for carrying out this project and Drs. Suzanne Lemieux, Daniel Oth, Pierre Talbot and Peter Tijssen, members of the graduate committee responsible for the Master's program in Virology and Immunology at the time of my admission, for accepting to enroll me in the program.

I would like to express great appreciation and gratitude to Dr. Pierre Talbot as my thesis director for his constant interest and dedication regarding all aspects of the research performed as well as the writing of the thesis itself, for his careful guidance and for many constructive discussions.

Much gratitude is expressed to Mrs. Francine Lambert for her orientation regarding the techniques and for helpful advice.

I would like to thank the personnel and the researchers of the Centre de Recherche en Virologie for their generosity in providing additional support, particularly Drs. Claire Simard, Gilles Lussier and Pierre Payment, as well as Ms. Ginette Godin and Ms. Micheline Letarte.

Gratitude is also expressed to Mr. André Levac and Ms. Ginette Larose for their aid in preparing the illustrations.

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