DETECTION OF MHV-A59 RNA BY IN SITU HYBRIDIZATION

Ehud Lavi, Donald H. Gilden, Maureen K. Highkin, and Susan R. Weiss

The Multiple Sclerosis Research Center of the Wistar Institute of Anatomy and Biology and the Departments of Neurology and Microbiology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104 USA

INTRODUCTION

We have shown that MHV-A59 causes a chronic demyelinating disease in C57BL/6 mice.[1] Although active demyelination can be detected as long as five months post inoculation, it has been difficult to detect viral antigens by immunofluorescence and thus far impossible to demonstrate replication of infectious virus in chronically infected mice. Thus we have used in situ hybridization of CNS and liver samples with radiolabeled virus-specific DNA probes to investigate the state of the genome in these tissues. In this paper we report experiments in which we start to address the question of whether or not viral RNA can be detected in the CNS and liver of these chronically infected mice and, if so, how much and in which cell types.

MATERIALS AND METHODS

Virus and Animals

MHV-A59, obtained from Dr. J. Leibowitz, was plaque purified three times in mouse 17CL-1 cells and grown at a low multiplicity of infection (m.o.i.). Stock virus contained $2x10^7$ plaque forming units (pfu)/ml. Certified MHV-free 4-6 week old C57BL/6 mice (Jackson Laboratories) were inoculated intracerebrally (i.c.) with $3x10^3$ pfu of virus in 0.03 ml. of PBS containing 0.75% BSA. At different intervals post infection (PI) mice were anesthetized with ether and perfused with 20 ml. of PBS followed by 20 ml. of periodate-lysin-paraformaldehyde (PLP) solution.[2] Brain, spinal

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cord and liver were removed and immersed in PLP overnight at 4°C, dehydrated and embedded in paraffin. Ten um sections were cut and collected on microscope slides which had been previously coated with Denhardt's solution and acetylated as previously described.[3] Infected and uninfected 17CL-1 mouse cells were harvested approximately 16 hours after infection with MHV-A59 (m.o.i.=1) when a maximum cytopathic effect (CPE) was observed and deposited by cytocentrifugation on microscope slides which had been previously treated as described for tissues above.

Preparation of MHV-A59 Probe

MHV-A59 DNA sequences, cloned into the bacterial plasmid pBR322, were used as probes for viral nucleic acids. cDNA was synthesized using as template either purified genome RNA or poly(A)-containing intracellular RNA from MHV-A59 infected cells, using oligo$(dT)_{12-18}$ as primer and reverse transcriptase as previously described except that all four nucleotide triphosphates were at 0.2mM concentrations.[4] After denaturation, the cDNA was copied into double-stranded DNA with E. coli polymerase I, the duplex was treated with S1 nuclease and deoxycytidine tails were added to the 3' ends with terminal transferase. This tailed DNA was hybridized with deoxyguanosine tailed, Pst-1 cleaved pBR322 and these hybrids were used to transform HB101 all essentially as described by Gough et al.[5] Colonies containing viral inserts were selected by two rounds of hybridization with virus-specific cDNA.[6] The sizes of viral inserts as estimated by migration in agarose gels of Pst-1 digested plasmid DNAs were from 400-1800 base pairs with the majority in the 400-600 base pair range. Clones synthesized from genome RNA were named pBRg1, etc., and those from intracellular RNA were named pBRc1, etc. The plasmid DNAs pBRg344 and pBRc8 were tritium-labeled by nick translation with DNA polymerase I at 14°C for two hours.[7] The specific activity of these probes was approximately $3x10^7$ cpm/ugDNA.

In Situ Hybridization

The probe was applied onto MHV-infected and uninfected mouse tissue sections and tissue culture cells at a concentration of $1\text{-}1.5x10^5$ cpm/1.13 cm^2 and processed as described for detection RNA[3]. The hybridization mixture contained mouse 17CL-1 cell nucleic acids which was necessary to eliminate non-specific binding of probes to tissue samples.

RESULTS

Characterization of Virus-Specific Cloned DNAs

In order to detect viral nucleic acids in mouse CNS samples, we wanted to use cloned probes representing as much of the viral

genome as possible. The MHV-A59-specific clones that we obtained (as described in Materials and Methods) were synthesized using oligo(dT) as a primer and thus would be expected to represent the 3' end of the genome RNA. However, we wanted to map them more precisely against the genome. Since the MHV intracellular virus-specific RNAs form a nested set, all overlapping at the 3' end, we used these RNAs for mapping of the cloned DNAs. Thus, plasmid DNAs from selected clones were nick translated with ^{32}P-dCTP[7] and hybridized to Northern blots containing intracellular RNA from uninfected and MHV-infected 17CL-1 cells. All cloned DNAs tested hybridized only with RNA from infected cells and to all seven virus-specific subgenomic RNAs (data not shown). This shows that all clones must map at least partially within the 3' nucleocapsid (N) gene which is encoded by the smallest RNA. All cloned DNAs cross-hybridized with each other except for pBRg344 (data not shown). Since clone pBRg334 is too long to map to the 3' side of the other clones, it probably maps to the 5' side and encompasses the E1 gene as well as part of N. We have used these data to generate a tentative map of these clones as shown in Figure 1. The sequences represented in pBRg344 and pBRc8 together represent approximately 12% of the MHV-A59 genome. We used DNA from both of these clones as a virus-specific probe.

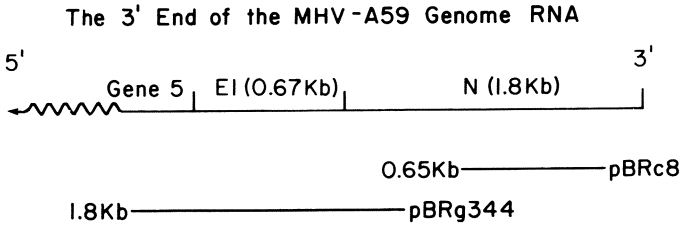


Fig. 1. The 3' end of the MHV-A59 genome RNA. The genes at the 3' end are designated N for nucleocapsid and E1 for the small MHV plycoprotein. The positions of two cloned DNA fragments are shown below.

Detection of MHV-A59 in 17CL-1 Infected Cells

As a positive control for the specificity of the probe in the detection of MHV-A59 RNA by *in situ* hybridization, we used infected 17CL-1 cells. MHV-A59 RNA was detected at maximal CPE (Figure 2A) in the cytoplasm of all cells, especially in giant syncytia. Uninfected 17CL-1 cells did not hybridize with the probe (Figure 2B).

Detection of MHV-A59 During Acute Infection in Mice

MHV-A59 RNA was detected in all three mice that were inoculated (i.c.) with $3x10^3$ pfu and sacrificed 5 days PI. In the liver, viral RNA was detected mostly in the cytoplasm of hepatocytes (Figure 3A) at the periphery of foci of inflammation and necrosis. The histological findings were compatible with acute hepatitis.

In the brain, viral genome was found at day 5 PI mostly in the white matter, but also in the meninges, cerebral cortex, thalamus and brain stem (data not shown). The histological findings were compatible with mild meningoencephalitis.

In the spinal cord, viral RNA was found at day 5 PI within the cytoplasm of cells (Figure 4A) in both the white and gray matter. The number of infected cells was smaller than in the brain. The histological finding was mild inflammation of spinal meninges and parenchyma.

Liver, brain and spinal cord sections from mock infected mice that were processed for *in situ* hybridization in parallel did not hybridize with the probe (Figures 3C and 4C).

Detection of MHV-A59 in Chronically Infected Mice

In the search for MHV-A59 RNA in chronically infected mice three mice were sacrificed 10 months PI and the organs were processed for *in situ* hybridization. MHV-A59 RNA was detected in the liver of one mouse in small foci of 3-4 hepatocytes each (Figure 3B). In spinal cord sections of two mice, viral genome was also detected within the cytoplasm of a few small round cells in the white matter (Figure 4B). It is impossible on the basis of morphology alone to classify the infected cells. It is also difficult to say whether the infected area also has pathological changes since the fixation procedure for *in situ* hybridization produces some myelin destruction. In both liver and CNS samples there were less foci of cells containing viral sequences during chronic infection

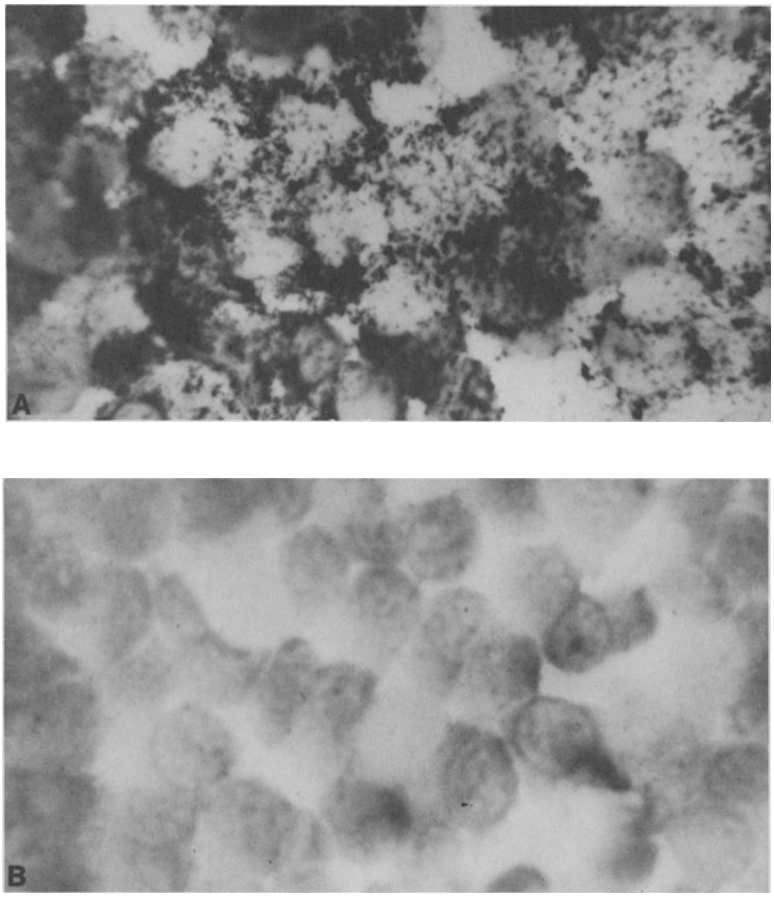
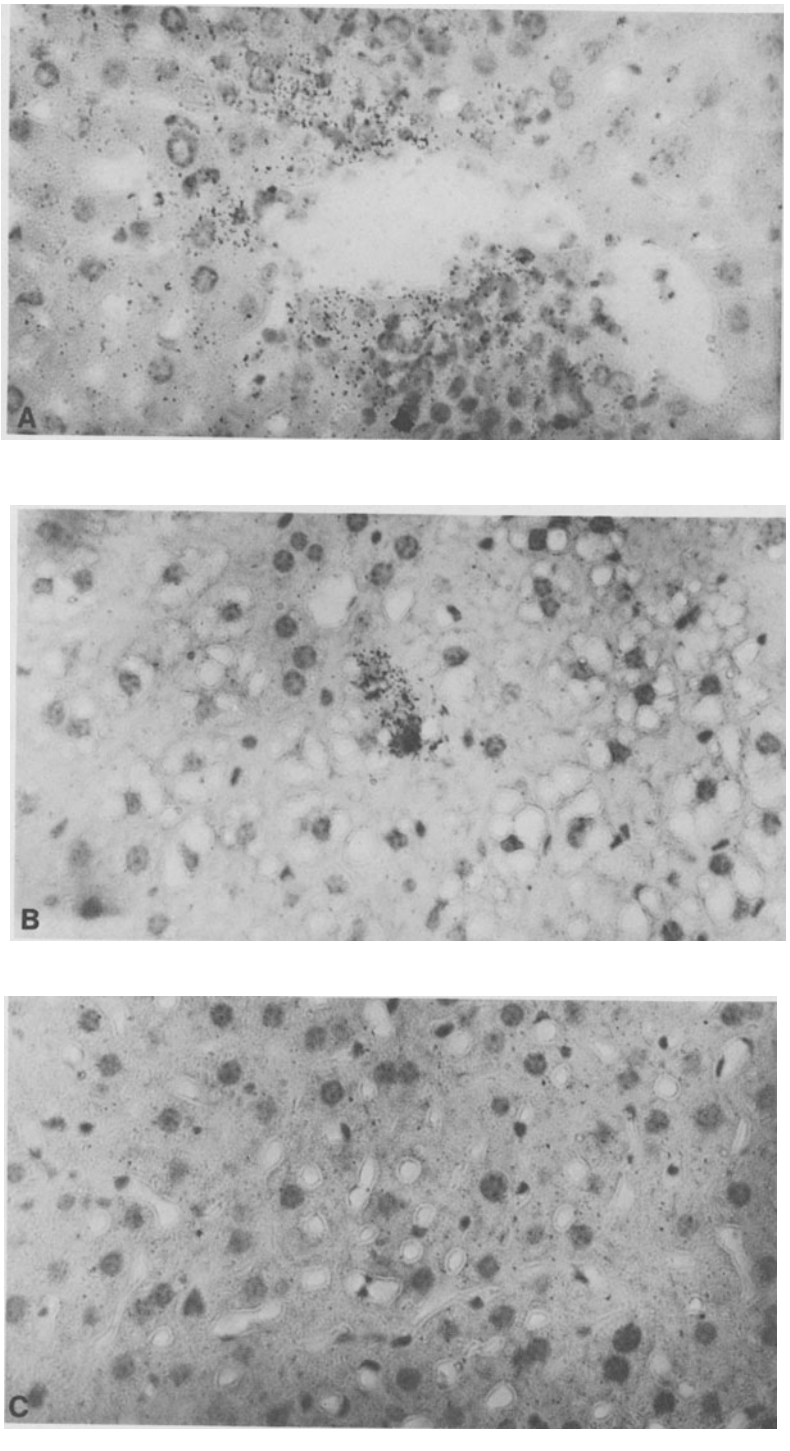


Fig. 2. MHV-A59 RNA detected by in situ hybridization with an H^3-labeled specific probe and autoradiographically exposed for one week. H&E x385.
A) MHV-A59 infected 17CL-1 cells (m.o.i.=1), harvested and fixed 16 hours PI at maximal cytopathic effect. Silver grains of positive hybridization are seen in the cytoplasm of all cells.
B) Uninfected 17CL-1 cells processed in paralled. No hybridization is seen.

as compared to acute infection. MHV-A59 could not be found in the liver, brain or spinal cord of control mock infected mice (Figures 3C and 4C).

Fig. 3. MHV-A59 RNA detected by *in situ* hybridization with an H^3-labeled specific probe and autoradiographically exposed for one week. H&E x385

A) Liver section from a mouse 5 days after i.c. inoculation with 3 $x10^3$ pfu MHV-A59. Viral RNA is seen in the cytoplasm of hepatocytes adjacent to a portal vein.
B) Liver section from a mouse 10 months after i.c. inoculation with $3x10^3$ pfu of MHV-A59. Viral RNA is seen in the cytoplasm of hepatocytes.
C) Liver section from a mock infected mouse processed in parallel. No hybridization is seen.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B
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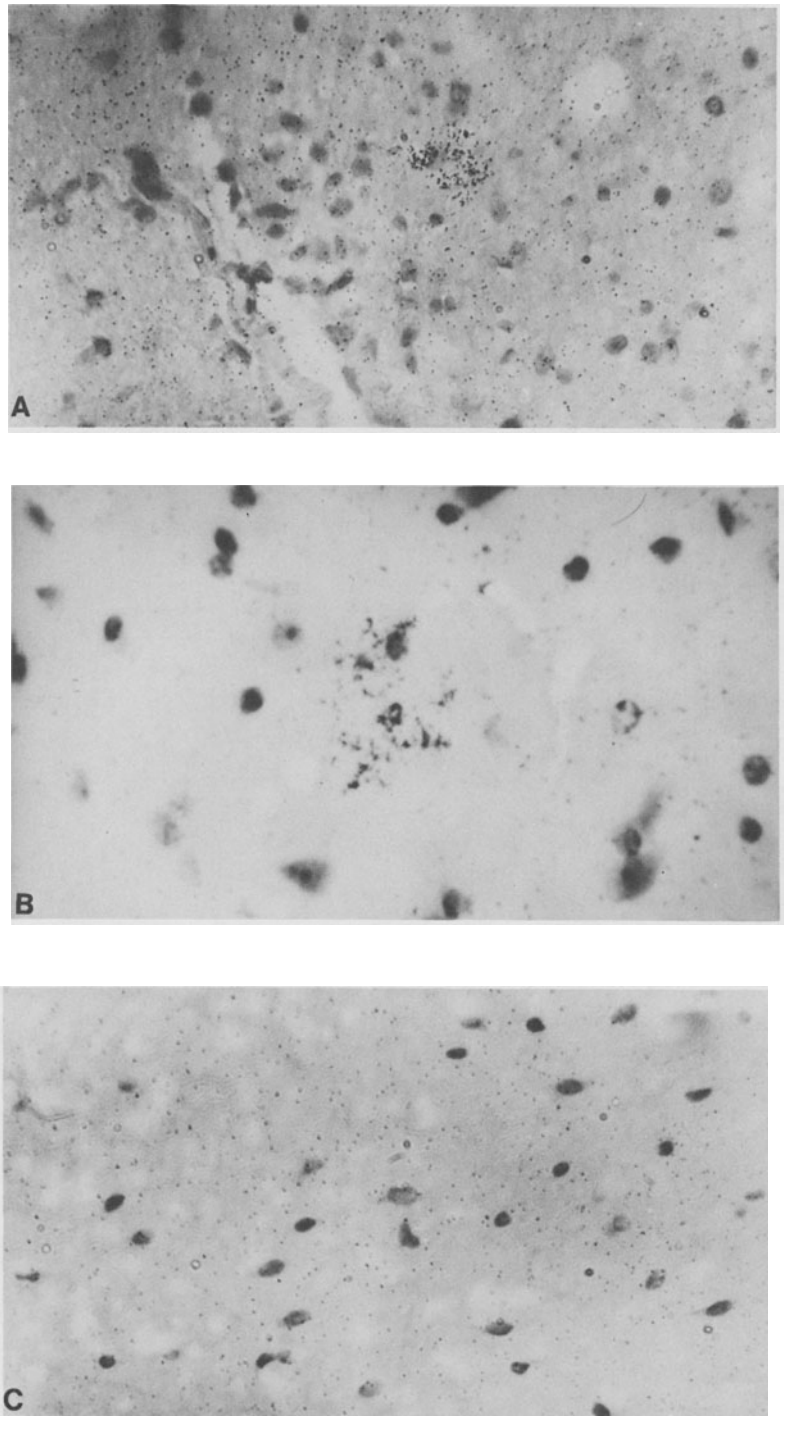
Fig. 4. MHV-A59 RNA detected by in situ hybridization with an H^3-labeled probe and autoradiographically exposed for one week. H&E x385.

A) Spinal cord section from a mouse 5 days after i.c. inoculation with $3x10^3$ pfu of MHV-A59. Viral RNA is detected in a few cells in the anterior columns of white matter.
B) Spinal cord of a mouse 10 months after i.c. inoculation with $3x10^3$ pfu of MHV-A59. Viral RNA is detected in a few white matter cells near the anterior horn.
C) Spinal cord section from a mock infected mouse processed in parallel. No hybridization is seen.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DISCUSSION

Viral persistence is a possible explanation for a broad array of chronic diseases in animals and humans. Two examples of chronic diseases attributed to coronavirus persistence are MHV-3 chronic vasculitis and MHV-JHM chronic demyelinating disease. The two diseases differ in pathogenic mechanism and apparently in target cell. Nevertheless, viral persistence has been shown in both. MHV-3 was recovered from chronically infected mice[8] and infectious JHM could be recovered from chronically infected mice[9] and rats.[10]

In MHV-A59 infected mice that develop chronic demyelination, there is persistence of virus-specific nucleic acid. Whether or not persistence of a low level of virus as evidenced by viral nucleic acid sequences is the mechanism of chronic demyelination is not yet clear. In another model system, Theiler virus infection of mice, viral persistence[11] as well as an immune-mediated mechanism[12] has been shown to be involved in chronic demyelination.

We show here that MHV-A59 RNA persists in both the CNS and the liver while chronic pathological changes develop only in the CNS. Since the number of cells containing viral genome is very low in both tissues, a possible explanation for this phenomenon can be an essential difference between oligodendrocytes and hepatocytes. A single oligodendrocyte is responsible for covering several axons with myelin. Thus, the destruction of an individual oligodendrocyte is potentially more damaging than the destruction of a single hepatocyte, which has little pathological effect.

It has thus far been impossible to recover MHV-A59 from chronically infected mice. This may be related to the small number of cells containing viral RNA. The *in situ* hybridization technique has been shown to be a very powerful tool for the detection of small amounts of viral nucleic acid,[13] as our study reienforces. It is still unclear whether a small amount of infectious virus is present.

The number of grains seen in chronically infected tissue cells is similar to the number of grains detected in acutely infected cells. This suggests that there is not a large difference in the amount of viral RNA per cell in the tissues from acutely or chronically infected animals, and that there is not a significant block in the amount of total viral transcription during persistent infection.

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