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Persistence of Mouse Hepatitis Virus A59 RNA in a Slow Virus Demyelinating Infection in Mice as Detected by In Situ Hybridization

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Mouse hepatitis virus strain A59 produces chronic central nervous system demyelination in rodents. As late as 6 months after intracerebral inoculation of mice 4 to 6 weeks old, when infectious virus cannot be recovered and viral antigens cannot be detected in the central nervous systems and livers of these animals, primary demyelination is still evident. Using cloned virus-specific DNAs and the highly sensitive and specific technique of in situ hybridization, we have detected low levels of mouse hepatitis virus A59 RNA in the central nervous systems and livers of mice 10 months after inoculation. We suggest that viral persistence may play a role in mouse hepatitis virus A59-induced chronic demyelination.

Mouse hepatitis virus (MHV) is a member of the coronavirus group, a group of positive-stranded, enveloped RNA viruses that produce both acute and chronic diseases in various animal species and account for about 15% of common colds in humans (10). The neurotropic strains of MHV, JHM and A59, produce chronic central nervous system (CNS) demyelination in rodents (9, 14). Elucidation of the mechanism of induction of virus-induced demyelination in these animal systems may be useful in understanding the etiology and pathogenesis of human demyelinating diseases such as multiple sclerosis.

The outcome of MHV-A59 infection of 4- to 6-week-old C57BL/6 mice depends on the dose and the route of inoculation (8). When inoculated intracerebrally with 1 50% lethal dose (3,000 PFU), all the mice develop a biphasic disease. Acute disease, consisting of severe hepatitis and mild meningoencephalitis, develops during week 2 postinfection (p.i.) and is resolved by week 3 p.i. Chronic disease, consisting of demyelinating lesions in the brain and spinal cord, begins 3 to 4 weeks after inoculation and lasts for at least 6 months (9). Infectious virus can be recovered from mice only during the acute phase, but traces of viral antigen can still be detected by immunofluorescence until 3 months p.i. By 6 months p.i., viral antigen cannot be detected, but demyelination is still evident (9). To determine whether chronic demyelination of MHV-A59 is associated with persistence of the viral genome, the technique of in situ hybridization was used.

The probes for the in situ hybridization experiments were synthesized by the radiolabeling of cloned DNAs representing parts of the MHV-A59 genome. Virus-specific cDNAs were cloned into the bacterial plasmid pBR322 by standard techniques (4). Briefly, cDNA was synthesized by using reverse transcriptase to copy polyadenylic acid-containing, virus-specific RNA with oligodeoxythymidylic acid$_{12-18}$ as primer. The cDNA was converted to double-stranded DNA, treated with S1 nuclease, tailed with oligodeoxycytidylic acid, and inserted into oligodeoxyguanidylic acid-tailed, *Pst*I-cleaved pBR322 (S. P. Wilczynski, C. J. Budzilowicz, and S. R. Weiss, submitted for publication). The clones obtained were mapped by hybridization with virus-specific mRNAs and by cross-hybridization with other cloned virus-specific DNAs. The probes we used here were synthesized by nick translation of plasmid DNA from two clones. These two clones, g344 (1,800 base pairs) and c8 (650 base pairs), were found to be nonoverlapping and to represent 12% of genome RNA sequences specifically in the three 3′ genes of MHV, i.e., *N* (the nucleocapsid), *E1* (the small glycoprotein), and gene 5 (unknown gene product). This is shown schematically in Fig. 1. Since the MHV genome is expressed as a nested set of seven mRNAs all overlapping at the 3′ ends, the 3′ portions of the genome are represented on mRNAs encoding the more 5′ genes. Thus, as we have previously shown, probes representing the 3′ end of genome RNA detect all the viral mRNAs as well as the genome (15).

To verify the specificity of the probe for in situ hybridization, the cloned DNAs were nick translated (12) with [^{3}H]TTP and were hybridized to uninfected and MHV-A59-infected 17CL-1 mouse fibroblasts (Fig. 2). The probe hybridized specifically to infected cells; background hybridization with uninfected cells was minimal after 6 days of autoradiographic exposure (the time needed to detect sequences in infected tissues). When cells were treated with RNase, hybridization with the probe showed only background activity. This was carried out to demonstrate that hybridization was to RNA, as would be expected for a cytoplasmic RNA virus. Probes derived from pBR322 plasmid DNA alone did not hybridize to either infected or uninfected cells (Fig. 2).

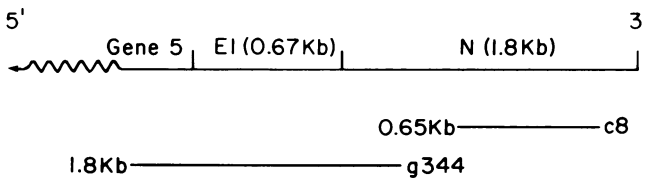

FIG. 1. Schematic diagram of the positions of the MHV-A59 cloned sequences at the 3′ end of the genome RNA. The approximate sizes as measured by electrophoresis in agarose gels and map positions as determined by hybridization with virus-specific mRNAs and other cloned DNA fragments are indicated, along with the MHV-A59 genome RNA.

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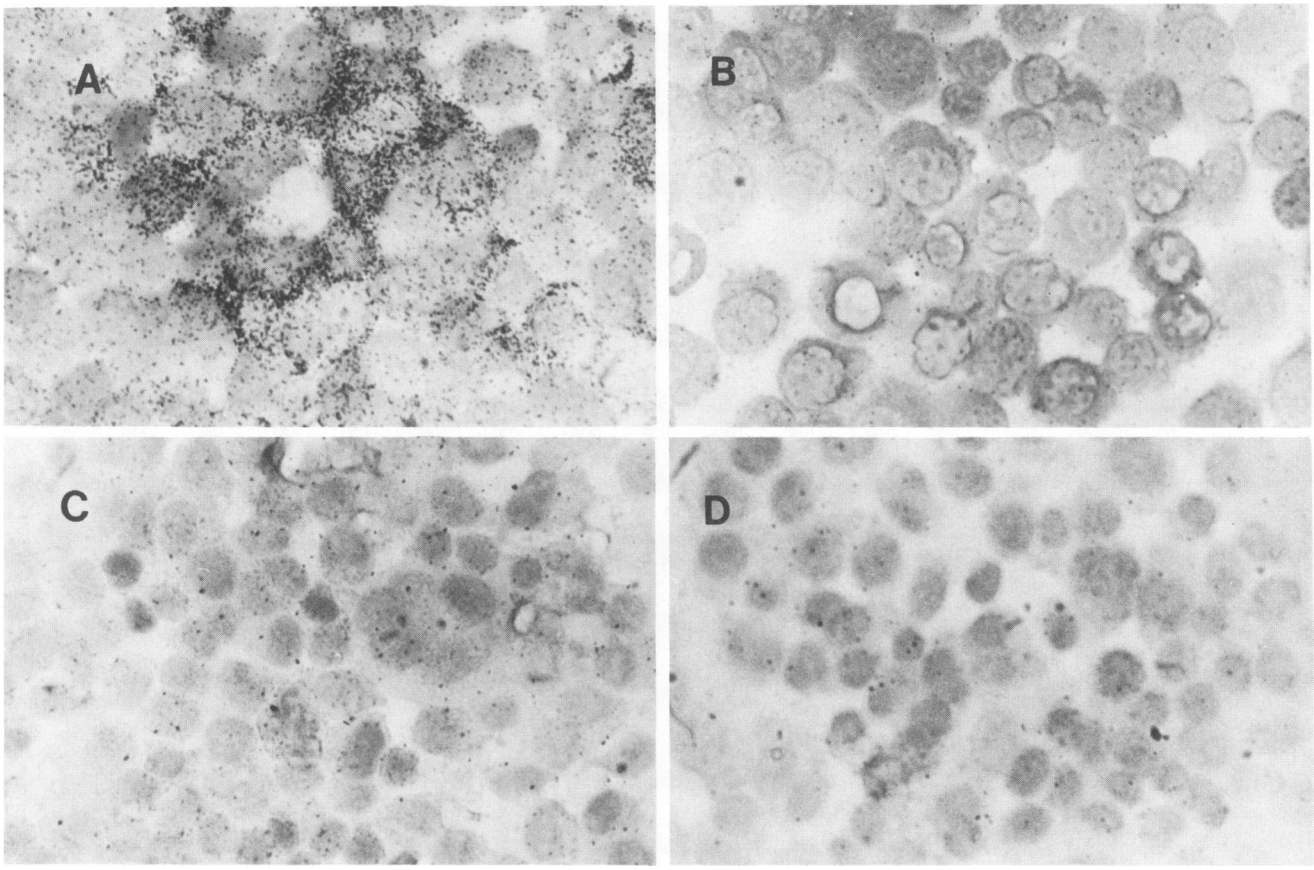

FIG. 2. Detection of MHV-A59 sequences in cultured cells by in situ hybridization. 17CL-1 cells that had been infected with MHV-A59 at a multiplicity of infection of 1 or had been mock infected were harvested at 16 h p.i. when maximum cytopathic effect was observed and were deposited by cytocentrifugation on microscope slides which had been previously coated with Denhardt solution and acetylated (1). The cells were then fixed and hybridized for 48 h with MHV-A59 cloned DNAs labeled with [^{3}H]TTP by nick translation (specific activity, 3×10^7 cpm/μg of DNA) at a probe concentration of 1×10^5 to 1.5×10^5 cpm/μg^2 and processed essentially as described (1). 17CL-1 cell RNA was included in the prehybridization mixture to eliminate nonspecific binding of probes to samples. Hybridization was assayed by exposure to NTB-2 Kodak emulsion for 6 days. (A) Positive hybridization was seen in the cytoplasm of infected cells. No hybridization was detected in (B) uninfected 17CL-1 cells processed in parallel, (C) infected cells treated with RNase before hybridization as in panel (A), or (D) infected cells treated as in (A) and hybridized with a probe derived from wild-type pBR322 plasmid DNA. Hematoxylin and eosin staining; magnification, ×400.

At various stages after inoculation with MHV-A59, mice were sacrificed, and tissues were processed and hybridized with the virus-specific DNA probe as described in the legend to Fig. 3. MHV-A59 RNA was detected in all three acutely infected mice that were examined. In the brain, viral genome was found mostly in the white matter but also in the meninges, cerebral cortex, thalamus, and brain stem. A mild meningoencephalitis was seen pathologically. In the spinal cord, viral RNA was found within the cytoplasm of cells both in the white and gray matter (Fig. 3A). There were fewer infected cells than in the brain. Inflammation of spinal meninges and parenchyma was seen. The probe did not hybridize with spinal cord (Fig. 3C), brain sections, or liver (Fig. 4C) from similarly processed mock-infected mice. In the liver, viral RNA was detected mostly in the cytoplasm of hepatocytes at the peripheries of foci of inflammation and necrosis (Fig. 4A). Acute hepatitis was seen pathologically.

Five chronically infected mice were sacrificed 10 months after infection with MHV-A59. In the spinal cords of two mice, viral RNA was detected within the cytoplasm of a few small round glial cells in the white matter (Fig. 3B). Viral RNA was detected in the liver of two other mice in small foci of three or four hepatocytes (Fig. 4B). Cytoplasmic vacuolization of hepatocytes was seen in these areas. In both liver and CNS samples, there were fewer foci of cells containing viral sequences during chronic infection than during acute infection. The occurrence of positive foci in chronically demyelinated versus acutely infected mice, as well as the prevalence of positive foci along the neuraxis, will be analyzed in detail in future experiments.

Five other infected mice were sacrificed at 10 months p.i. and were analyzed for infectious virus and viral antigen in the CNS. For the infectious virus assay, brain homogenates were plaqued on 17CL-1 cells under conditions that would have detected 10^2 PFU per brain (9). Viral antigen was assayed on tissue slices by indirect immunofluorescence by using mouse anti-A59 hyperimmune serum and fluorescein-conjugated goat anti-mouse immunoglobulin G (9). Results for all animals were negative for infectious virus and viral antigen.

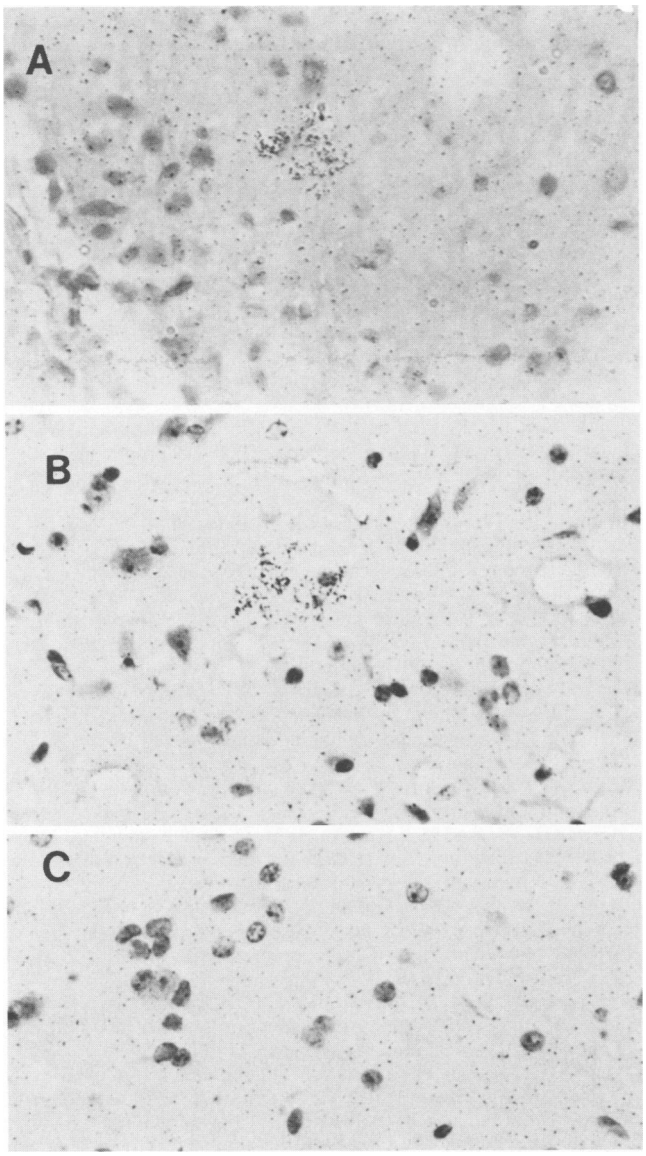

FIG. 3. Detection of MHV sequences in CNS tissue samples by in situ hybridization. Certified MHV-free 4- to 6-week-old C57BL/6 mice (Jackson Laboratories) were inoculated intracerebrally with 3 $\times 10^3$ PFU of virus in 0.03 ml of phosphate-buffered saline containing 0.75% bovine serum albumin. At different times p.i., mice were anesthetized with ether and perfused with 20 ml of phosphate-buffered saline followed by 20 ml of periodate-lysine-paraformaldehyde solution (11). Brain, spinal cord, and liver were removed and immersed in the same fixative solution overnight at 4°C and then were dehydrated and embedded in paraffin. Sections (10 μm) were cut and collected on microscope slides which had been previously treated with Denhardt solution and acetylated (1). The tissues were then fixed, hybridized, and exposed as described in the legend to Fig. 2. (A) Spinal cord section from a mouse 5 days p.i. Viral RNA is detected in a few cells in the anterior column white matter adjacent to inflammatory response. (B) Spinal cord section from a mouse 10 months p.i. Viral RNA is detected in the cytoplasm of a few white matter cells near the anterior horn. (C) Spinal cord section from a mouse 5 days after mock-infection. No hybridization is seen. Hematoxylin and eosin staining; magnification, ×400.

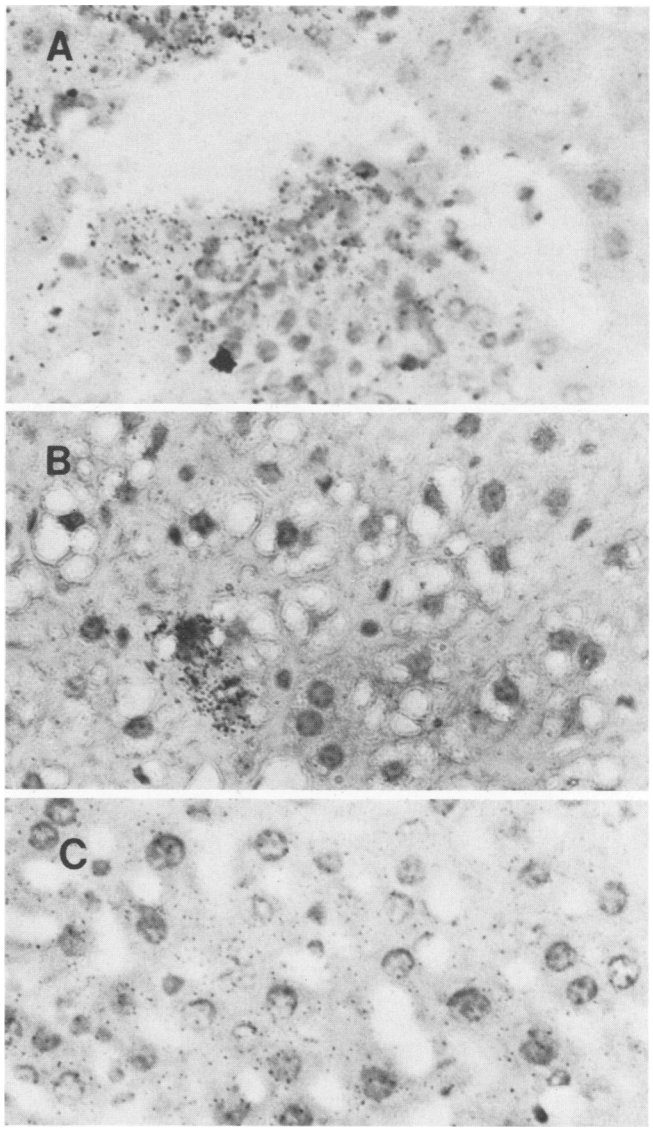

FIG. 4. Detection of MHV sequences in liver tissue samples by in situ hybridization. Tissue sample preparation and hybridization were as described in the legends to Fig. 2 and 3. (A) Liver section from a mouse 5 days p.i. Viral RNA is seen in the cytoplasm of hepatocytes adjacent to a portal vein. (B) Liver section from a mouse 10 months p.i. Viral RNA is seen in the cytoplasm of hepatocytes. (C) Liver section from a mouse 5 days after mock infection. No hybridization is seen. Hematoxylin and eosin staining; magnification, × 400.

It has been hypothesized that the chronic demyelination induced by MHV-JHM is due to a cytolytic infection of the myelin-producing cells, oligodendrocytes (3, 7). In the chronic MHV infections described here, viral nucleic acids persisted in the CNS and liver of mice for months after it was possible to recover infectious virus or even detect viral antigens. Although virus-specific sequences were found both in white and grey matter during acute infection, viral sequences were found only in white matter during chronic

infection, suggesting that oligodendrocytes may be the target cells for chronic infection. Similarly, in Theiler virus-induced demyelination of mice, virus-specific sequences were detected in grey and white matter during acute infection but were found only in the white matter during chronic demyelinating disease (2). However, whether the virus-specific sequences in white matter in the coronavirus and Theiler virus infections are restricted to oligodendrocytes remains to be determined.

The probes used in our experiments represent only 3′ portions of genomic RNA. Thus, the viral sequences detected could represent partial genomes. We are currently determining whether the rest of the genome is present by hybridizing with cloned probes representing 5′ regions of the genome. It seems likely that the entire genome is present and replicating at a low level because low levels of infectious virus have been recovered from mice with MHV-3-induced chronic vasculitis (13) and MHV-JHM *ts*8-induced chronic demyelinating disease (6).

Haase et al. (5) postulated that agents causing slow and persistent infections must satisfy two conditions: (i) the lytic destruction of tissue by the interaction of virus with host cells must be mitigated to allow the host to survive for long periods, and (ii) viral genetic information must be stably conserved in cells in which expression of viral antigens is sufficiently curtailed to allow the infected cells to escape detection and elimination by the immune system. Thus, MHV-A59 infection in mice satisfies both these criteria. A likely hypothesis to explain MHV-A59 persistence in the CNS and liver is that virus replicates in certain permissive cells (presumably hepatocytes and oligodendrocytes) at a low level and that acute exacerbations of the disease are prevented by the immune system.

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