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Temperature-Sensitive Mutants of Mouse Hepatitis Virus Strain A59: Isolation, Characterization and Neuropathogenic Properties

MARCK J. M. KOOLEN,* ALBERT D. M. E. OSTERHAUS,† GIJSBERT VAN STEENIS,† MARIAN C. HORZINEK,* AND BERNARD A. M. VAN DER ZEIJST*,[1]

*Institute of Virology, Veterinary Faculty, State University, 3508 TD Utrecht, and †National Institute of Public Health (RIV), 3720 BA Bilthoven, The Netherlands

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Twenty 5-fluorouracil-induced temperature-sensitive (ts) mutants of mouse hepatitis virus strain A59 were isolated from 1284 virus clones. Mutants were preselected on the basis of their inability to induce syncytia in infected cells at the restrictive temperature (40°) vs the permissive temperature (31°). Of these mutants, only those with a relative plating efficiency 40°/31° of 3×10^{-3} or smaller were kept. Virus yields at 40° compared to 37° and 31° (leakiness) were determined. Most mutants (16) were RNA^-, i.e., unable to synthesize virus-specific RNA at the restrictive temperature. The other four were RNA^+. No qualitative differences were detected in the virus-specific RNAs in cells infected with RNA^+ ts-mutants, both at 31° and 40°. Virus-specific proteins present in cells infected with ts-171 (RNA^-) and the RNA^+-mutants (ts-43, ts-201, ts-209, and ts-279) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of immunoprecipitates. No qualitative differences in the pattern of virus-specific cellular proteins were detected among the mutants except for an additional polypeptide of about 46,000 daltons in ts-209-infected cells. Finally, the neuropathogenic properties of eight of the mutants were investigated. Whereas 10^2 PFU of wild-type virus injected intracerebrally killed 50 to 100% of 4-week-old Balc/c mice within 1 week, the mutants were highly attenuated. A dose of 10^5 PFU lead to no or transient disease. However, 4 weeks after infection with ts-342, ts-43, or ts-201 obvious histological changes were observed in brain and spinal cord of clinically healthy mice.

INTRODUCTION

We have undertaken to isolate temperature-sensitive (ts) mutants of mouse hepatitis virus strain A59 (MHV-A59), with the expectation that these mutants will be useful for further studies on the replication and pathogenesis of this virus. MHV-A59 is a member of the family Coronaviridae, a group of lipid-enveloped viruses with a single strand of infectious RNA of about 6×10^6 molecular weight (Tyrrell *et al.*, 1978; Ter Meulen *et al.*, 1981).

In the last few years, considerable progress has been made in elucidating the replication strategy and structure of coronaviruses (for reviews see Ter Meulen *et al.*, 1981; Siddell *et al.*, 1982). A characteristic feature of the coronavirus replication strategy is that seven, or six in the avian system, virus-specific RNAs are synthesized in the infected cell. The largest one is the intracellular form of genome; the others are a nested set of subgenomic mRNAs with common 3'-proximal sequences (Spaan *et al.*, 1981, 1982; Wege *et al.*, 1981; Lai *et al.*, 1981; Leibowitz *et al.*, 1981; Cheley *et al.*, 1981; Weiss and Leibowitz, 1981; Stern and Kennedy, 1980a, b). RNAs 3, 6, and 7 have been shown to encode the three major virion proteins (Siddell *et al.*, 1980; Rottier *et al.*, 1981a; Cheley *et al.*, 1981; Leibowitz *et al.*, 1982). RNAs 1 and 2 of MHV encode nonstructural proteins (Leibowitz *et al.*, 1982; Siddell *et al.*, 1981). ts mutants will be useful to identify the functions of these and other nonstruc-

[1] To whom reprint requests should be addressed.

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tural proteins and for the study of the transcription mechanism. In addition they might be helpful to elucidate the process of virus maturation. Moreover, we were interested to know whether the pathogenic properties of MHV-A59 would change after the induction of mutations into the genome. A59 virus is a hepatotropic strain of MHV, causing extensive hepatic necrosis and leading to death within a week (Manaker *et al.*, 1961). When injected intracerabrally, the virus leads to encephalitis (Robb *et al.*, 1979). For the neurotropic strain of mouse hepatitis virus, MHV-4(JHM), a clear difference was observed between wild-type virus and mutant ts-8 (Haspel *et al.*, 1978). Whereas wild-type virus produced a rapid and fatal encephalomyelitis, the mutant induced a prolonged infection of the central nervous system accompanied by demyelination and mice survived. Immunocytochemical studies *in vivo* have shown that ts-8 has a restricted tropism for neurons and replicates mostly in oligodendrocytes (Knobler *et al.*, 1981).

MATERIALS AND METHODS

Cells and virus. Mouse L cells and Sac(−) cells were grown in Dulbecco's modified Eagle medium containing 10% fetal calf serum (DMEM-10% FCS) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin. The preparation of virus stocks of MHV-A59 has been described previously (Spaan *et al.*, 1981).

Virus was plaque-titrated on L cells (Spaan *et al.*, 1981) using an overlay with 1.5% Bacto-agar (Difco, Detroit, Mich.) in DMEM-1% FCS unless otherwise indicated. Plaques were read after one (37°, 40°) or three (31°) days. A subline of L cells obtained from F. Lehmann-Grube (Hamburg, West Germany) was used. With these cells plaques could be read sooner than reported previously (Spaan *et al.*, 1981); also plaque titers were five- to sevenfold higher.

For most experiments Sac(−) cells were grown in 35-mm tissue culture dishes and infected at densities of 1 to 2×10^6 using 50 PFU/cell in 0.3 ml DMEM-3% FCS. After 1 hr this inoculum was replaced by 1 ml DMEM-10% FCS.

Mutagenesis. Sac(−) cells were infected with doubly cloned MHV-A59 wild-type virus at a multiplicity of infection (m.o.i.) of 10. One hour postinfection (p.i.) inocula were removed and replaced by 10 ml of DMEM-10% FCS supplemented with 150 μg/ml 5-fluorouracil. Virus was harvested at 16 hr p.i. (37°) and stored at −70°. Mutagenized virus stocks had an infectivity titer of 1.2×10^4 PFU/ml (37°) a reduction of about 1000 times compared to the untreated control.

Selection of mutants. Mutagenized virus stocks diluted as to give 15 plaques in a 35-mm tissue culture dish were plaqued on L cells at 31°. In order to facilitate subsequent elution of virus an overlay of 0.4% agarose (Merck-Schuchard, Darmstadt, Germany) was used. After 3 days, well-isolated plaques were picked using the back of a Pasteur pipet. Plaques were then disrupted by vortexing in 0.5 ml DMEM-3% FCS resulting in a virus suspension containing about 10^5 PFU/ml. With this suspension parallel cultures, at 31° and 40°, of Sac(−) cells were infected. About 10^4 PFU (0.1 ml) was added to 2×10^5 cells grown in 0.5 ml DMEM-10% FCS in the 16-mm wells of tissue cultures clusters (Costar, Cambridge, Mass.). The cells at both temperatures were observed daily for the appearance of syncytia. Those virus clones that caused syncytium formation 3 days p.i. at 31° but not at 40° 2 days p.i. were considered as potential ts mutants. Each potential mutant was tested to confirm its ts nature by plaquing the medium of the 31° culture on L cells at the restrictive and permissive temperature. Only those mutants having a plating efficiency 40°/31° of 3×10^{-3} or lower were used.

The leakiness of the mutants was determined by infecting 5×10^5 Sac(−) cells grown in 16-mm wells of tissue culture clusters with wild-type virus or ts mutants in 0.1 ml of DMEM-3% FCS at an m.o.i. of 50 at 31°. After 1 hr the inocula were removed and replaced by 0.5 ml DMEM-10% FCS, and incubation was continued at 31°, 37°, or 40° until respectively 16, 12, and 9 hr p.i. Virus titers in the culture

medium were determined by plaque titration on L cells at 31°.

Preparation of virus stocks of ts mutants. Stocks of the mutant viruses were prepared by infecting 75-cm^2 tissue culture flasks of Sac(−) cells with about 10^3 PFU of the original virus clones eluted from plaques. Virus was adsorbed for 1 hr in 1.5 ml DMEM-3% FCS at 31° before 10 ml DMEM-10% FCS was added. Stocks were harvested after an additional 48–72 hr at the permissive temperature and stored at −70°.

High-titered virus stocks needed for infection of cells at high m.o.i. were prepared by infecting roller flasks (1200 cm^2) containing about 2×10^8 Sac(−) cells with the ts mutants at 0.2 PFU/cell in 25 ml DMEM-3% FCS for 1 hr at 31°. Inocula were removed and 100 ml DMEM-10% FCS was added and incubation was continued at 31°. The virus was harvested at 48 p.i. and precipitated from the medium with polyethylene glycol (Spaan *et al.*, 1981). The resulting virus pellet was resuspended in 1 to 2 ml DMEM-3% FCS per roller flask.

Determination of the kinetics of virus formation and the synthesis of virus-specific RNAs at 31° and 40° in wild-type virus-infected cells. Sac(−) cells in 35-mm tissue culture dishes were infected as described above. After adsorption, the cells were washed once with PBS and medium was added. Samples from the culture fluid were taken at various times p.i. and plaque-titrated. The kinetics of virus-specific RNA synthesis were determined by following the incorporation of [^{3}H]uridine into actinomycin D-treated Sac(−) cells as described previously (Spaan *et al.*, 1981).

Analysis of virus-specific proteins in cells infected with ts mutants. Sac(−) cells in 35-mm dishes were infected at 31° and replicate cultures were incubated at 31° and 40°. Seven hours p.i. (40°) or 13 hr p.i. (31°) the cells were washed twice with PBS and labeled with 1 ml methionine-deficient MEM, supplemented with 5% FCS and 10 μCi [^{35}S]methionine (~1000 Ci/mmol, The Radiochemical Centre, Amersham, England) until 9 hr p.i. (40°) or 16 hr p.i. (31°). After the labeling period, cells were washed with PBS and lysed in 0.15 ml lysis buffer: 0.5% Triton X-100, 0.5% 1,5-naphthalene-disulfonate-disodium salt, and 2 m*M* phenylmethylsulfonyl fluoride in TES buffer (20 m*M* Tris, pH 7.4, 1 m*M* EDTA, 100 m*M* NaCl). The lysates were centrifuged for 5 min at 10,000 *g* and processed directly or kept at −70°.

Virus-specific proteins were immunoprecipitated by incubating 30 μl of the clarified samples with 10 μl mouse anti-MHV-A59 serum. The serum was obtained from Balb/c mice surviving an experimental MHV-A59 infection (Spaan *et al.*, 1981). After overnight incubation at 4°, 0.2 vol of 3 *M* KCl was added, followed by 0.1 ml of a 10% (v/v) suspension of formaldehyde-treated *Staphylococcus aureus* (Kessler, 1975) in TES buffer containing 0.1% Triton X-100. Incubation was continued for 30 min before immune complexes were collected by centrifugation for 2 min at 10,000 *g* and washed three times with 0.15 ml of TES buffer containing 0.1% Triton X-100. Finally, the adsorbed proteins were dissolved in 50 μl electrophoresis sample buffer (10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.001% bromophenol blue). To avoid aggregation of the E1 protein species (Sturman, 1977), samples were not boiled prior to electrophoresis in 15% acrylamide–0.085% bisacrylamide gels (Rottier *et al.*, 1981b). Gels were loaded with 10,000 cpm of radioactivity of the immunoprecipitated virus-specific proteins synthesized at 31°, which were compared with material from an equal number of cells incubated at 40°. Proteins were visualized by fluorography on preflashed Kodak XS film or Fuji RX film at −70° (Laskey *et al.*, 1975).

Analysis of intracellular virus-specific RNAs. Sac(−) cells in 35-mm tissue culture dishes were infected under standard conditions at 31°, refed with medium, and shifted to 40° or kept at 31°. From 7 hr p.i. (31°) or 3 hr p.i. (40°) on, the cells were incubated in medium supplemented with 1 μg/ml actinomycin D. One hour later [^{3}H]uridine (50 μCi/ml, 28 Ci/mmol; The Radiochemical Centre) was added and the cells were labeled until 16 hr p.i. (31°) or 9 hr p.i. (40°). After this period, they were

washed and RNA was isolated by phenol extraction as described previously (Jacobs *et al.*, 1981). RNAs were analyzed by electrophoresis in 6 *M* urea-containing agarose gels (Rottier *et al.*, 1981a).

Pathology in mice. The neuropathogenic properties of the ts mutants were determined by intracerebral injection of groups of four 4-week-old Balb/c mice (Centraal Proefdierenbedrijf TNO, Zeist, The Netherlands) with 10^5 PFU in 0.1 ml PBS. Before injection, animals were bled and sera were checked for the absence of anti-MHV antibodies in a plaque reduction assay. Control animals received 0.1 ml PBS without virus. The mice were kept in isolators and were observed daily for clinical disfunctions. Four weeks after injection, they were bled and fixed by total perfusion through the heart with about 7 ml of 4% formaldehyde in phosphate-buffered saline. Brains and spinal cords were embedded in paraffine, and sections were stained with haematoxylin-eosin.

RESULTS

Choice of the Restrictive (40°) and Permissive (31°) Temperature, Growth Kinetics of Wild-Type MHV-A59 at These Temperatures

Wild-type virus plaqued equally well on mouse L cells at 31°, 37°, and 40°. Also in Sac(−) cells similar virus titers were reached at the three temperatures (Fig. 1A; Spaan *et al.*, 1981) but whereas maximum virus yields in the medium were obtained at 8 hr at 40°, about 16 hr was needed at 31°. As described before (Spaan *et al.*, 1981), virus yield at 37° is maximal at 10 hr p.i. The accumulation of virus-specific RNA was determined by measuring the incorporation of [^{3}H]uridine into actinomycin D-treated Sac(−) cells. Figure 1B shows that it closely followed the curve for virus release. On the basis of this experiment virus-specific RNA synthesis in mutants was studied from 8 to 16 hr at 31° and from 4 to 9 hr at 40°.

5-Fluorouracil was chosen as a mutagen under conditions where it induced 1-2% ts mutants (Van Berlo *et al.*, 1980), a proportion at which multiple ts lesions are not likely to occur.

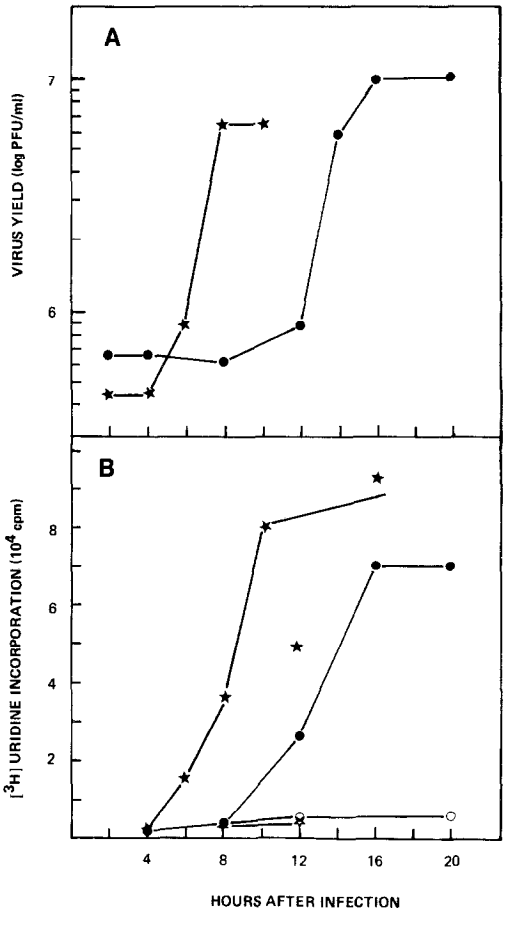

FIG. 1. Kinetics of virus growth (A) and viral RNA synthesis (B) at 31° (●) and 40° (★) in Sac(−) cells infected with wild-type MHV-A59. Cells were infected at 50 PFU/cell and at various times p.i. samples were taken for virus titration. The synthesis of viral RNA was measured by following the incorporation of [^{3}H]uridine added to the medium at 2 hr p.i. Actinomycin D (1 μg/ml) was present from 1 hr p.i. on. Open symbols represent mock-infected cultures.

A prescreening of ts mutants was made on the basis of their inability to induce syncytia at 40°. This resulted in 148 potential mutants out of a total of 1284 isolated plaques. The final selection was made by plaquing potential mutants at the permissive and restricted temperature. Only those mutant having a relative plating efficiency 40°/31° of 3×10^{-3} or less were

TABLE 1

BIOLOGICAL AND BIOCHEMICAL PROPERTIES OF MOUSE HEPATITIS VIRUS STRAIN A59 ts MUTANTS

Mutant	Virus titer (PFU/ml) at 31° [a]	Efficiency of plating at 40°/31° [b]	Progeny yield (PFU/ml)			RNA synthesis at 40°	Protein synthesis at 40°
			31°	37°/31° [c]	40°/31° [c]		
ts$^+$	5×10^8	1	2.8×10^8	0.600	0.600	+	+
ts-8	1×10^8	2×10^{-6}	6.5×10^7	0.077	0.005	−	−
ts-43	5×10^6	2×10^{-5}	7×10^5	0.016	0.009	+	−
ts-88	1×10^8	8.3×10^{-4}	7.6×10^7	0.526	0.013	−	−
ts-145	3×10^8	8.3×10^{-6}	2.8×10^8	0.002	0.001	−	−
ts-169	9×10^8	3×10^{-3}	1×10^7	0.180	0.022	−	−
ts-171	1.5×10^7	$<2.2 \times 10^{-6}$	2.3×10^7	0.009	0.001	−	−
ts-195	8×10^7	1.3×10^{-5}	9.2×10^7	0.003	0.002	−	−
ts-201	2×10^7	2.5×10^{-4}	9×10^5	0.089	0.017	+	+
ts-209	5×10^6	1×10^{-3}	2×10^5	0.500	0.050	+	+
ts-261	6×10^8	$<5.5 \times 10^{-7}$	2.7×10^8	0.003	0.003	−	−
ts-267	8×10^7	1.3×10^{-4}	1.3×10^8	0.005	0.003	−	−
ts-276	8×10^7	3×10^{-4}	1.2×10^7	0.041	0.023	−	−
ts-290	1×10^8	4×10^{-4}	6.7×10^7	0.010	0.005	−	−
ts-299	1.5×10^8	$<2.2 \times 10^{-7}$	1×10^6	0.001	0.008	−	−
ts-329	3×10^7	7×10^{-4}	1.2×10^6	0.042	0.042	−	−
ts-342	2.5×10^8	4×10^{-6}	1×10^7	1.000	0.001	−	−
ts-364	5×10^8	1.7×10^{-3}	2.4×10^8	0.133	0.003	−	−
ts-379	5×10^7	$<6.6 \times 10^{-6}$	7×10^6	0.001	0.001	+	+
ts-380	1.2×10^8	3.8×10^{-7}	3.5×10^7	0.571	0.010	−	−
ts-396	6.5×10^7	7.7×10^{-6}	1×10^7	0.005	0.003	−	−

[a] Plaque titer of virus stocks on L cells.
[b] Ratio of plaque titers at 40° and 31°. For nonleaky mutants this approximates the reversion frequency.
[c] Leakiness at 37° and 40°.

kept. This resulted in 20 mutants, i.e., 1.6% of the initial number of virus clones.

A number of biological characteristics of the mutants are summarized in Table 1. Among them are the yields after one step growth at 40°, 37°, and 31° which allow calculations of the leakiness of a mutant. Knowledge of virus growth at 37°, the approximate body temperature of mice (Weir, 1947) is relevant for the interpretation of *in vivo* infection experiments.

Synthesis at the Restrictive Temperature of Virus-Specific RNAs in Cells Infected with Mutants

The ts mutants were tested for their ability to synthesize RNA at the permissive and restrictive temperature. Infected Sac(−) cells were labeled with [^{3}H]uridine in the presence of actinomycin D. Cells infected with wild-type virus incorporated comparable amounts of [^{3}H]uridine at the permissive and restrictive temperature resulting in a 30-fold stimulation of RNA synthesis over the background in mock-infected cells. Four of the ts mutants were able to synthesize virus-specific RNA at both the restrictive and the permissive temperature, giving a 10- to 25-fold stimulation compared to actinomycin D-treated, mock-infected cells. The other 16 ts mutants were RNA$^-$, i.e., virus-specific RNA-synthesis at 40° was twofold or less above the background in mock-infected cells.

Cells infected with the RNA$^+$ mutants (ts-43, ts-201, ts-209, and ts-379) were phenol extracted and virus-specific RNAs from these cells were analyzed. As shown in Fig. 2, no qualitative differences were observed between the set of virus-specific RNAs infected in these cells compared to the RNAs present in cells infected with wild type vi-

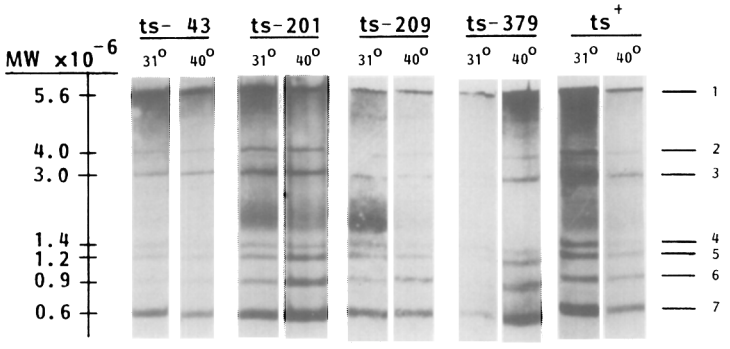

FIG. 2. Analysis by agarose gel electrophoresis of virus-specific RNAs extracted from Sac(−) cells infected with wild type MHV-A59 (ts$^+$) or RNA$^+$ ts mutants grown at 31° and 40°. Gels were loaded with equal amounts (50,000 cpm) of radioactivity of the virus-specific RNAs synthesized at 31°, which were compared with RNA extracted from an equal number of cells incubated at 40°. Fluorographs were exposed for 7 days.

rus. The RNA$^-$ mutants were also analyzed to see whether there was still some preferential synthesis of one or more of the RNAs; none was detected.

Virus-Induced Proteins in Cells Infected with ts Mutants

RNA$^+$ mutants were tested for their ability to synthesize proteins at the permissive and restrictive temperature. Virus-specific intracellular proteins were labeled with [^{35}S]methionine and immunoprecipitated. As described previously (Rottier *et al.*, 1981b), Sac(−) cells infected with wild-type MHV-A59 contain gp150, the precursor of the virion proteins gp90/180/E2, the nucleocapsid protein pp54/N, from which during the immunoprecipitation procedure usually two smaller polypeptide species arise (Rottier *et al.*, 1981a), and the three forms of the matrix protein E1, p24, gp25.5, gp26.5 (Fig. 3). These same proteins were observed in cells infected with mutants ts-201, ts-209, and ts-379 both at the permissive and restrictive temperature. However, ts-209-infected cells contained an extra 46K protein band, which could be a degradation product of the nucleocapsid protein generated during immunoprecipitation. The fact that it is also found in straight cell lysates (not shown) argues against this, however.

A protein of about 120K was present in cells infected with ts mutants at 31°. Its nature is still unclear. It does not comigrate with p110, the unglycosylated precursor of gp150, found in tunicamycine-treated cells (data not shown).

Surprisingly, ts-43 although RNA$^+$ was unable to synthesize viral proteins at 40°, although some nucleocapsid protein might be present (Fig. 3).

Biological Effects of ts Mutants

Wild-type MHV-A59 is highly pathogenic for mice as was described before (Manaker *et al.*, 1961). We have found that 50 to 100% of 4-week-old Balb/c mice infected intraperitoneally or intracerebrally with this virus die within 1 week from hepatitis or encephalitis, respectively. Ts mutants are highly attenuated in their neuropathogenic properties. Of the mutants tested so far (ts-43, ts-169, ts-201, ts-209, ts-276, ts-299, ts-342, and ts-379), 10^5 PFU injected intracerebrally did not result in any clinical illness, with the exception of ts-169 which caused transient clinical symptoms 6 to 7 days p.i. Full details will be published elsewhere, but relevant at this moment is that histological sections of the central nervous system of mice infected with ts-342 sacrificed 4 weeks after infection showed, compared with control animals, obvious histological changes (Fig. 4),

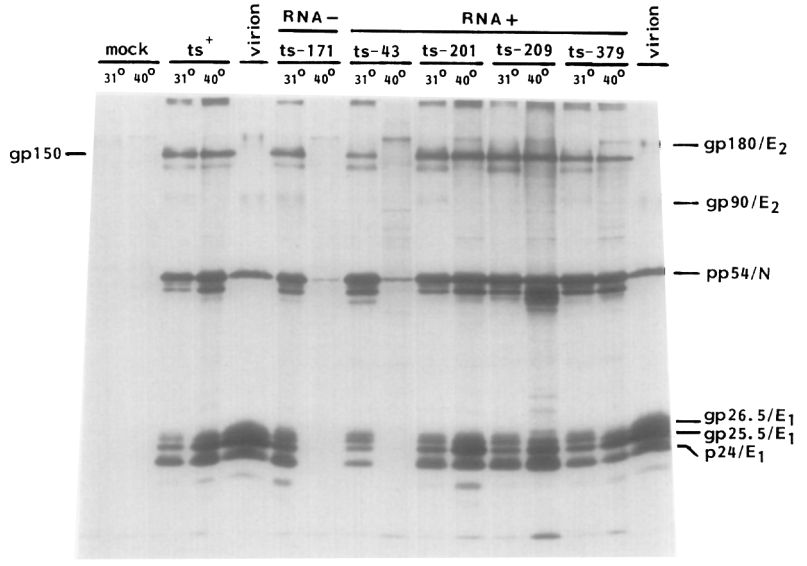

FIG. 3. Electrophoretic analysis of virus-specific proteins present in cells infected with RNA⁻ mutants (ts-43, ts-201, ts-209, and ts-379), mutant ts-171 and wild type (ts⁺) of MHV-A59. [^{35}S]Methionine labeled virus was prepared according to Rottier *et al.* (1981b).

although the mice were clinically healthy. Patchy areas of spongy degeneration were observed in the white matter of the spinal cord. These areas were not restricted to any particular tract. Similar areas were seen scattered throughout the brain stem, where also mainly the white matter appeared to be involved. Occasionally structures resembling swollen degenerating axons and macrophages were noted in the affected areas. Mild microglial reaction, and especially in the brain stem, astroglial reaction accompanied the lesions. There was otherwise little or no inflammatory reaction, except for a rare perivascular cuff.

In some of the mice, dilation of the ventricular system, especially of the lateral ventricles, occurred, with partial disappearance of the ventricular ependyma and rarefaction of the periventricular cortical aera. Similar, although less extensive, pathological changes were found in sections of the central nervous system obtained from mice infected with ts-43 and ts-201.

DISCUSSION

This paper reports the isolation and initial characterization of a set of MHV-A59 ts mutants. Only 4 of the 20 isolated mutants were able to induce virus-specific RNA in infected cells at the restrictive temperature. Assuming a random distribution of mutations over the genome and estimating about 55% of the coding capacity specifying replicative functions (Van der Zeijst *et al.*, 1981), this is a surprisingly small proportion. It might reflect a greater sensitivity of the viral polymerase(s) to mutations compared to the viral structural proteins. A similar large proportion of RNA⁻ mutants was found in a set of ts mutants of MHV-JHM (Leibowitz *et al.*, 1982).

The RNA⁻ mutants will be useful for the study of the viral genes involved in viral RNA synthesis. Our preliminary unpublished experiments have demonstrated that in MHV-A59 at least five complementation groups are involved in viral RNA synthesis. This unexpectedly high number is in agreement with data from Leibowitz *et al.* (1982) who recently described six complementation groups for RNA⁻ mutants of MHV-JHM.

Since protein synthesis is unlikely to occur without RNA synthesis, only RNA⁺ mutants were tested for their ability to

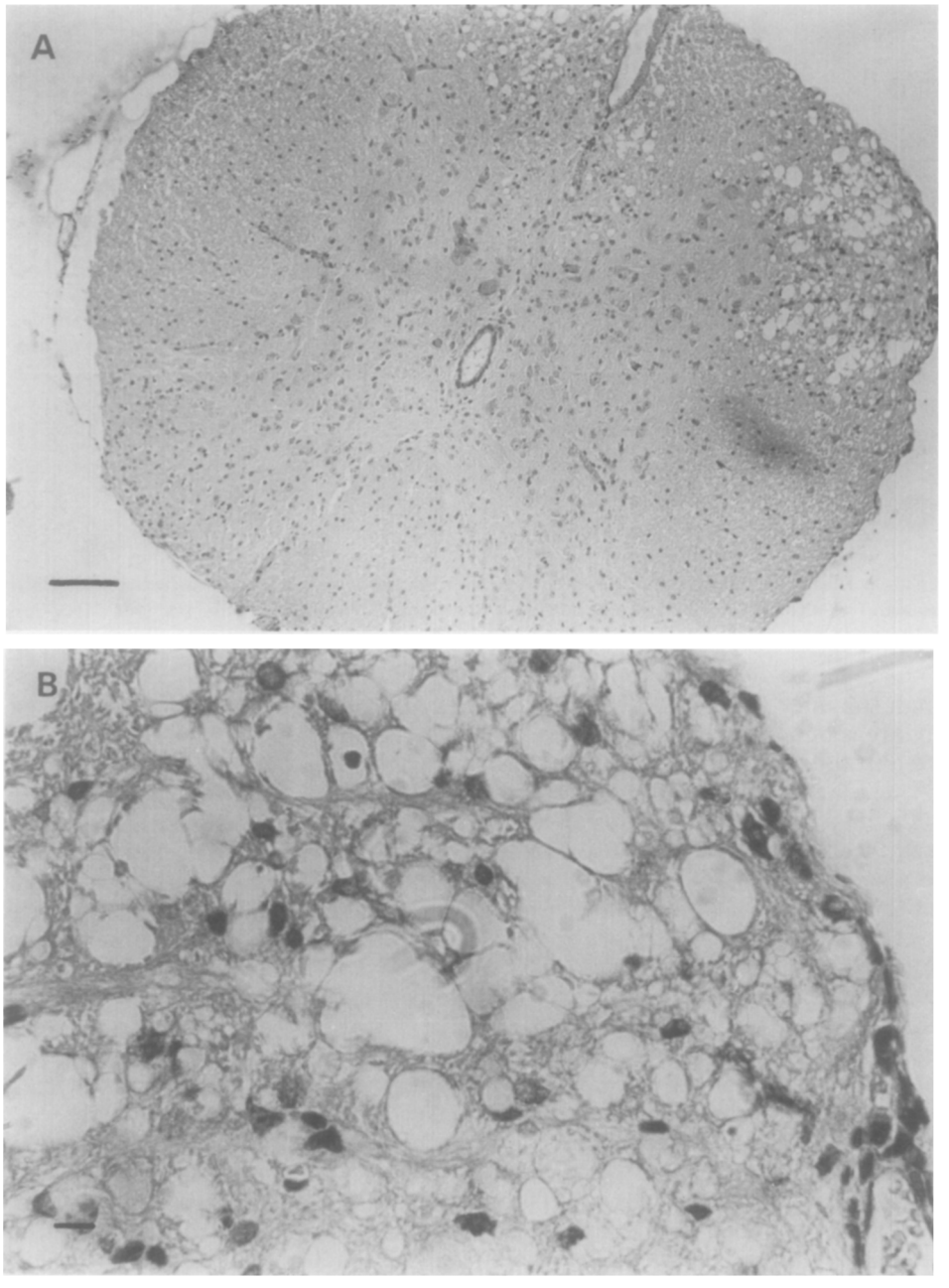

FIG. 4. Focal lesions in the spinal cord of a clinically healthy Balb/c mouse 4 weeks after intracerebral inoculation with 100,000 PFU of ts-342. Sections were stained with hematoxylin–eosin. The scale bar is 100 μm in A and 10 μm in B, a magnification of the upper right part of A. C is a control from mock-infected mice.

induce synthesis of virus-specific polypeptides at the permissive and restrictive temperature. Three of these mutants (ts-201, ts-209, ts-379) induced all viral proteins, both at the restrictive and the permissive temperature. However, one mutant, ts-43, was unable to induce virus-specific proteins in cells at the restrictive temperature with the possible exception of a small amount of nucleocapsid protein. It

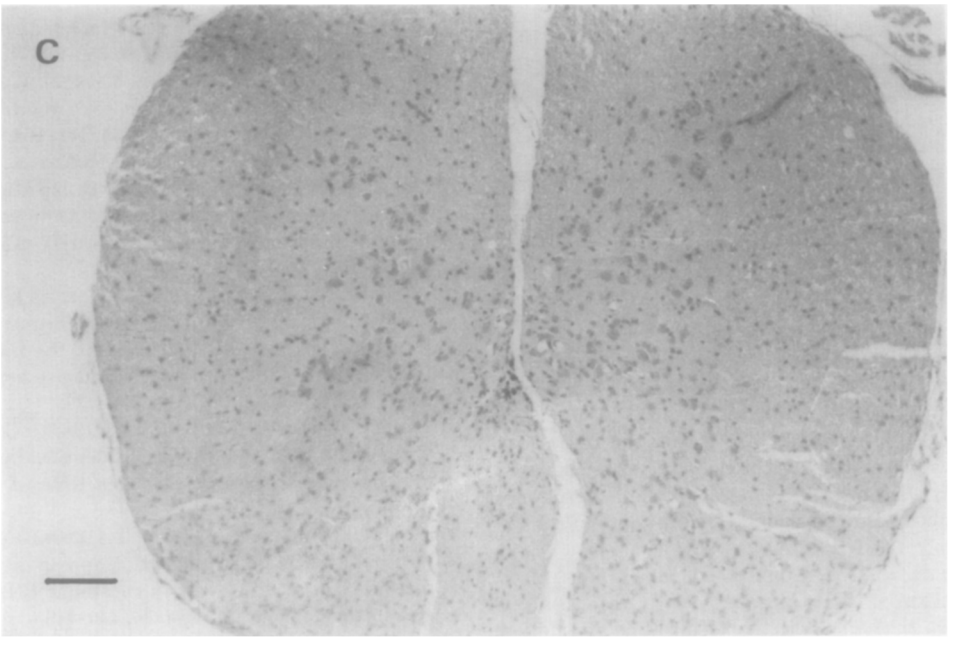

FIG. 4—*Continued.*

will be of interest to see whether the RNAs made at 40° are translatable *in vitro*.

There is some confusion about the pathogenic properties of MHV-A59. Some investigators have reported that the virus has a very low virulence and a weak pathogenicity (Lai and Stohlman, 1981; Robb *et al.*, 1979). This might be explained by assuming that these investigators were working with a nonpathogenic variant of the original virus isolated by Manaker *et al.* (1961). A more likely explanation, however, is that the mice used for the pathogenic studies had antibodies against mouse hepatitis virus (Robb *et al.*, 1979). We have always checked our mice for the absence of anti-MHV antibodies by a plaque reduction assay, before they were used for pathogenic studies. Doing so we were able to confirm the observation of Manaker *et al.* (1961) that the virus is indeed highly pathogenic and virulent, when injected intracerebrally or intraperitoneally. We show here that attenuated virus has drastically attenuated pathogenic properties and is able to cause more prolonged infection of the central nervous system. In this respect there seems to be little difference between so-called hepatotropic MHV-A59 and neurotropic MHV-JHM. A detailed study on the time course of the development of the lesions in the central nervous system and the cells involved is in progress.

We assume that the ts defects are due to a change in one single nucleotide, although during mutagenesis inevitably other non-ts lesions will have been induced. Therefore, it is not sure whether the ts lesion itself or additional mutations are responsible for the altered pathogenic properties of the virus. The high leakiness of ts-342 at 37° suggests that in this case additional mutations are important. Infection experiments with revertants of the mutant can help to elucidate this point.

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REFERENCES

CHELEY, S., ANDERSON, R., CUPPLES, M. J., LEE CHAN, E. C. M., and MORRIS, V. L. (1981). Intracellular murine hepatitis virus-specific RNAs contain common sequences. *Virology* **112,** 596–604.

HASPEL, M. V., LAMPERT, P. W., and OLDSTONE, M. B. A. (1978). Temperature-sensitive mutants of mouse hepatitis virus produce a high incidence of demyelination. *Proc. Nat. Acad. Sci. USA* **75,** 4033–4036.

JACOBS, L., SPAAN, W. J. M., HORZINEK, M. C., and VAN DER ZEIJST, B. A. M. (1981). Synthesis of subgenomic mRNAs of mouse hepatitis virus is initiated independently: Evidence from UV transcription mapping. *J. Virol.* **39,** 401–406.

KESSLER, S. W. (1975). Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: Parameters of the interaction of antibody-antigen complexes with protein A. *J. Immunol.* **115,** 1617–1624.

KNOBLER, R. L., DUBOIS-DALCQ, M., HASPEL, M. V., CLAYSMITH, A. P., LAMPERT, P. W., and OLDSTONE, M. B. A. (1981). Selective localization of wild type and mutant mouse hepatitis virus (JHM strain) antigens in CNS tissue by fluorescence, light and electron microscopy. *J. Neuroimmunol.* **1,** 81–92.

LAI, M. M. C., BRAYTON, P. R., ARMEN, R. C., PUGH, C., and STOHLMAN, S. A. (1981). Mouse hepatitis virus A59: mRNA structure and genetic localization of the sequence divergence from hepatotropic strain MHV-3. *J. Virol.* **39,** 823–834.

LASKEY, R. A., and MILLS, A. D. (1975). Quantitative detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* **56,** 331–335.

LEIBOWITZ, J., DE VRIES, J. R., and HASPEL, M. V. (1982). Genetic analysis of murine hepatitis virus strain JHM. *J. Virol.* **42,** 1080–1087.

LEIBOWITZ, J. L., WEISS, S. R., PAAVOLA, E., and BOND, C. W. (1982). Cell-free translation of murine coronavirus RNA. *J. Virol.* **43,** 905–913.

LEIBOWITZ, J. L., WILHEMSEN, K. C., and BOND, C. W. (1981). The virus-specific intracellular RNA species of two murine coronaviruses: MHV-A59 and MHV-JHM. *Virology* **114,** 39–51.

MANAKER, R. A., PICZAK, C. V., MILLER, A. A., and STANTON, M. F. (1961). A hepatitis virus complicating studies with mouse leukemia. *J. Nat. Cancer Inst.* **27,** 29–51.

ROBB, J. A., BOND, C. W., and LEIBOWITZ, J. L. (1979). Pathogenic murine coronaviruses. III. Biological and biochemical characterization of temperature sensitive mutants of JHMV. *Virology* **94,** 385–399.

ROTTIER, P. J. M., SPAAN, W. J. M., HORZINEK, M. C., and VAN DER ZEIJST, B. A. M. (1981a). Translation of three mouse hepatitis virus strain A59 subgenomic RNAs in Xenopus laevis oocytes. *J. Virol.* **38,** 20–26.

ROTTIER, P. J. M., HORZINEK, M. C., and VAN DER ZEIJST, B. A. M. (1981b). Viral protein synthesis in mouse hepatitis virus strain A59-infected cells: Effect of tunicamycin. *J. Virol.* **40,** 350–357.

SIDDELL, ST., WEGE, H., and TER MEULEN, V. (1982). The structure and replication of coronaviruses. *Curr. Top. Microbiol. Immunol.* **99,** 131–164.

SPAAN, W. J. M., ROTTIER, P. J. M., HORZINEK, M. C., and VAN DER ZEIJST, B. A. M. (1981). Isolation and identification of virus-specific mRNAs in cells infected with mouse hepatitis virus (MHV-A59). *Virology* **108,** 424–434.

SPAAN, W. J. M., ROTTIER, P. J. M., HORZINEK, M. C., and VAN DER ZEIJST, B. A. M. (1982). Sequence relationships between the genome and the intracellular RNA species 1, 3, 6 and 7 of mouse hepatitis virus strain A59. *J. Virol.* **42,** 432–439.

STERN, D. F., and KENNEDY, S. I. T. (1980a). Coronavirus multiplication strategy. I. Identification and characterization of virus-specified RNA. *J. Virol.* **34,** 665–674.

STERN, D. F., and KENNEDY, S. I. T. (1980b). Coronavirus multiplication strategy. II. Mapping the avian infectious bronchitis virus intracellular RNA species to the genome. *J. Virol.* **36,** 440–449.

STURMAN, L. S. (1977). Characterization of a coronavirus. I. Structural proteins: Effects of preparative conditions on the migration of protein in polyacrylamide gels. *Virology* **77,** 637–649.

TER MEULEN, V., SIDDELL, S., and WEGE, H. (1981). "Biochemistry and Biology of Coronaviruses," Adv. Exp. Med. Biol., vol. 142. Plenum, New York.

TYRRELL, D. A. J., ALEXANDER, D. J., ALMEIDA, J. D., CUNNINGHAM, C. H., EASTERDAY, B. C., GARWES, D. J., HIERHOLZER, J. C., KAPIKIAN, A., MACNAUGHTON, M. R., and MCINTOSH, K. (1978). Coronaviridae: Second report. *Intervirology* **10,** 321–328.

VAN BERLO, M. F., HORZINEK, M. C., and VAN DER ZEIJST, B. A. M. (1980). Temperature-sensitive mutants of equine arteritis virus. *J. Gen. Virol.* **49,** 97–104.

VAN DER ZEIJST, B. A. M., HORZINEK, M. C., JACOBS, L., ROTTIER, P. J. M., and SPAAN, W. J. M. (1981). Messenger RNAs of mouse hepatitis virus A59: Isolation and characterization, translation in *Xenopus laevis* oocytes of RNAs 3, 6 and 7, UV target sizes of the transcription templates. *In* "Biochemistry and Biology of Coronaviruses" (V. Ter Meulen, S. G. Siddell, and H. Wege, eds.), pp. 209–225. Plenum, New York.

WEGE, H., SIDDELL, S., STURM, M., and TER MEULEN, V. (1981). Coronavirus JHM: characterization of intracellular viral RNA. *J. Gen. Virol.* **54,** 213–217.

WEIR, J. A. (1947). The temperature of the mouse in health and disease. *Proc. Iowa Acad. Sci.* **54,** 383–388.

WEISS, S. R., and LEIBOWITZ, J. L. (1981). Comparison of the RNAs of murine and human coronaviruses. *In* "Biochemistry and Biology of Coronaviruses" (V. ter Meulen, S. G. Siddell, and H. Wege, eds.), pp. 245–259. Plenum, New York.