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Characterization of Two Temperature-Sensitive Mutants of Coronavirus Mouse Hepatitis Virus Strain A59 with Maturation Defects in the Spike Protein

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Two temperature-sensitive (*ts*) mutants of mouse hepatitis virus strain A59, *ts*43 and *ts*379, have been described previously to be *ts* in infectivity but unaffected in RNA synthesis (M. J. M. Koolen, A. D. M. E. Osterhaus, G. van Steenis, M. C. Horzinek, and B. A. M. van der Zeijst, Virology 125:393–402, 1983). We present a detailed analysis of the protein synthesis of the mutant viruses at the permissive (31°C) and nonpermissive (39.5°C) temperatures. It was found that synthesis of the nucleocapsid protein N and the membrane protein M of both viruses was insensitive to temperature. However, the surface protein S of both viruses was retained in the endoplasmic reticulum at the nonpermissive temperature. This was shown first by analysis of endoglycosidase H-treated and immunoprecipitated labeled S proteins. The mature Golgi form of S was not present at the nonpermissive temperature for the *ts* viruses, in contrast to wild-type (wt) virus. Second, gradient purification of immunoprecipitated S after pulse-chase labeling showed that only wt virus S was oligomerized. We conclude that the lack of oligomerization causes the retention of the *ts* S proteins in the endoplasmic reticulum. As a result, *ts* virus particles that were devoid of S were produced at the nonpermissive temperature. This result could be confirmed by biochemical analysis of purified virus particles and by electron microscopy.

Coronaviruses (31) are enveloped viruses with a large single-stranded RNA genome of approximately 30,000 nucleotides. They infect a range of mammals and birds, but the individual members of the *Coronaviridae* usually have narrow host specificity (18). The best-studied coronavirus is mouse hepatitis virus (MHV), which serves as a model for the coronavirus replication cycle, including such aspects as entry, protein synthesis, RNA replication, and virus assembly.

Virus-specific components of MHV virions are four structural proteins and RNA; the envelope is derived from the intermediate compartment (IC), which is the site of budding (13). The largest of the structural proteins is the surface protein S, a membrane protein that forms the characteristic coronavirus spikes. S is responsible for attachment of virions to the host cell and plays a role in fusion of the viral envelope with the host cell membrane (33). Two other membrane proteins are important for virus particle formation and play a role in budding: the membrane protein M and the small membrane protein E. The fourth structural protein, N, encapsidates the viral RNA. Except E, the structural proteins of MHV have been studied extensively. Much is known about the synthesis and maturation of particularly S and M, initially from studies of cells infected with wild-type (wt) or variant viruses. By using a range of different expression systems in the absence of virus, these proteins were studied in greater detail by mutagenesis of their cloned genes (reviewed in references 5 and 28). This approach was also taken to determine the functions of the structural proteins, but these experiments have the disadvantage of not being in the context of natural infections, and much remains to be elucidated. Only the nucleocapsid gene is currently subjectable to mutagenesis on the genomic RNA by a targeted RNA recombination protocol (24, 25).

Our current state of knowledge of the synthesis of the MHV structural proteins can be summarized as follows (for recent reviews, see references 4, 15, and 28). Membrane proteins S and M (probably also E, but no data are available) are synthesized on the rough endoplasmic reticulum (ER). Cotranslational N-glycosylation of the 120,000-molecular weight (120K) precursor of S yields a 150K form. M is posttranslationally O-glycosylated to an approximately 25K M protein. The 150K S protein folds with a half-time of approximately 20 min to its native state (22) and then associates with M, most likely before it oligomerizes (20). S oligomers are probably trimers, in accordance with the properties reported for the surface protein of transmissible gastroenteritis virus (6). Oligomeric S-M complexes are transported out of the ER into the IC. Monomeric, misfolded S is retained in the ER. In the IC, nucleocapsids interact with the S-M complexes, and budding into the IC results in virions (13, 34). It is unknown whether nucleocapsids are preformed or associate during budding. Also, it should be noted that the E protein plays a crucial but to date unknown role in the budding process (2, 36). Virions are transported from the IC to the Golgi compartment, where S and M are further glycosylated to the 180K mature form of the spike protein and a range of M proteins. At the *trans* Golgi, virions are encapsulated into vesicles of the constitutive pathway, transported to the plasma membrane, and subsequently released. During transport, a portion of S180 (depending on the MHV strain) is cleaved into two 90K forms: S2, which remains membrane bound; and S1, which is associated to S2.

Our laboratory is interested in the functions of coronavirus MHV-A59 S in attachment to the receptor and fusion of the virus envelope with the host cell plasma membrane. In a previous investigation, we studied maturation and cleavage of expressed mutants of S, altered in the cleavage site. In addition, S proteins with mutations in the transmembrane se-

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quence were tested for their fusogenicity (1). Any definitive answers on the functions of S require studying the protein in infections using virions. The direct approach to such studies is to mutagenize the S gene on the viral genome. To date, however, introducing mutations into genes located internally on the genome is technically impossible because of the lack of an infectious cDNA clone. An alternative to such a clone would be to insert mutant S proteins transiently into virions by complementation of temperature-sensitive (*ts*) MHV mutants with defects in the synthesis or maturation of the surface protein. In this paper, we report as a first step the characterization of two such mutants of MHV-A59, selected from a panel of *ts* mutants isolated by Koolen et al. in 1983 (12).

MATERIALS AND METHODS

Cells and viruses. Mouse L cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal calf serum (FCS). Virus stocks of MHV-A59 *ts* mutants *ts*43 and *ts*379 were prepared as described previously (12). MHV-A59 wt, *ts*43, and *ts*379 were diluted in phosphate-buffered saline–DEAE–2% FCS and subsequently used to infect L cells at a multiplicity of infection (MOI) of 0.1. After 2 days of growth at 31°C, virus was harvested. Virus titers were determined at 31 and 39.5°C on L cells. MHV-A59 wt was prepared as described previously (17).

Isolation and analysis of viral RNAs. Dishes (10-cm^2 diameter) with L cells were infected in duplicate with MHV-A59 wt, *ts*43, or *ts*379 at an MOI of 10 for 1 h at 31°C. At $t = 1$, the inoculum was replaced by 1.5 ml of DMEM–3% FCS. One dish was placed at 31°C, and the duplicate was placed at 39.5°C. Intracellular RNA was isolated at $t = 17$ (31°C) or $t = 8$ (39.5°C) as described previously (32). RNA was separated on a denaturing 1% agarose gel as described previously (35). The gel was dried and hybridized to 100 ng of oligonucleotide 048 (35) which was 5′-end labeled with [γ-^{32}P]ATP (NEN-Dupont) and T4 polynucleotide kinase.

Isolation and analysis of viral proteins. Dishes (10-cm^2 diameter) with L cells were infected in duplicate with MHV-A59 wt, *ts*43, or *ts*379 at an MOI of 10 for 1 h at 31°C. At $t = 1$, the inoculum was replaced by 1.5 ml of DMEM–3% FCS; one dish was placed at 31°C, and one was placed at 39.5°C. At $t = 4$ (39.5°C) or $t = 9$ (31°C), the medium was replaced by 500 μl of DMEM lacking both cysteine and methionine. One hour later, 8 μl of Expres^{35}S label (10 μCi/μl; NEN) was added. Intracellular proteins were obtained by lysing the cells at $t = 13$ (31°C) or $t = 7$ (39.5°C) in 300 μl of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate). The lysates were cleared from nuclei and membrane fragments by centrifugation, and the proteins in supernatants were immunoprecipitated in the presence of Pansorbin (Calbiochem) with polyclonal antibody k134, directed against MHV-A59 whole virus, and separated on a 12.5% polyacrylamide gel as described previously (1).

For the analyses of the S protein, a portion of the cell lysates was immunoprecipitated with a mixture of two monoclonal antibodies (MAbs) against the spike protein of MHV-A59: A2.3 and A1.4, kindly provided by J. Fleming. Immunoprecipitation pellets were washed three times in RIPA buffer and dissolved in 50 μl of 50 mM Tris (pH 6.8)–0.25% sodium dodecyl sulfate. The samples were incubated at 95°C for 5 min and cleared by centrifugation for 2 min. Ten-microliter aliquots of the supernatants were mixed with 15 μl of 30 mM sodium acetate and incubated in the presence or absence of endoglycosidase H (endo-H; 1 mU/μl; Boehringer) for 16 h at 37°C. The samples were separated on a 12.5% polyacrylamide gel as described above.

Sucrose gradient purification of virus. Dishes (10-cm^2 diameter) with L cells were infected in duplicate with MHV-A59 wt, *ts*43, or *ts*379 at an MOI of 10 for 1 h at 31°C. At $t = 1$, the inoculum was replaced by 1.5 ml of DMEM–3% FCS; one dish was placed at 31°C, and the other was placed at 39.5°C. At $t = 3.5$ (39.5°C) or $t = 11$ (31°C), the medium was replaced by 500 μl of DMEM lacking cysteine and methionine. Thirty minutes later, 8 μl of ^{35}S-Trans-label (10 μCi/μl) was added. At $t = 14.5$ (31°C), the medium was replaced by 500 μl of DMEM containing 400 mM methionine and 400 mM cysteine. Virions released into the medium were finally collected at $t = 7$ (39.5°C) or $t = 16.5$ (31°C) and mixed with 500 μl of 20% sucrose in TESV (20 mM Tris [pH 7.4], 1 mM EDTA [pH 8.0], 100 mM NaCl). This mixture was loaded on top of a 20 to 50% linear sucrose gradient. The gradient was centrifuged for 16 h at 16,000 rpm at 4°C in an SW40Ti rotor, and 16 fractions of 690 μl were collected. The virus-containing fractions (particles migrated around a density of 1.18 g/ml) were located; three peak fractions were combined, and their volume was adjusted to 5 ml with TESV. Virus was pelleted by centrifugation in an SW55Ti rotor for 3 h at 35,000 rpm at 4°C. The resulting pellet was dissolved in 30 μl of Laemmli sample buffer and loaded directly on a 12.5% polyacrylamide gel.

Electron microscopy. Virus preparations were obtained by infection of 145-cm^2 dishes of L cells with an MOI of 10 at 31°C for 1 h. At $t = 1$, the inoculum was replaced by 15 ml of DMEM–3% FCS, and incubation continued at 39.5°C. At $t = 7$, the medium was harvested and diluted 1:1 with 45 ml of TESV–20% polyethylene glycol 6000. Virus was precipitated overnight at 4°C with mild stirring and then centrifuged for 30 min at 8,000 rpm at 4°C. The pellets were dissolved in 500 μl of TESV and mixed with 500 μl of 20% sucrose. This material was loaded on 20 to 50% linear sucrose gradients as described above. Labeled viruses, grown on 10-cm^2 dishes of L cells, were used as markers in parallel gradients.

One drop of purified virus was incubated for 1 min on a 400-mesh copper grid coated with Formvar, and then 1 drop of 2% phosphotungstic acid (pH 7.1) was added for 1 min. Phosphotungstic acid was removed, and the grids were examined with an electron microscope (Philips EM 410LS) at 80 kV.

Oligomerization analysis. Dishes (10-cm^2 diameter) with L cells were infected in duplicate with *ts*43, *ts*379, or MHV A59 wt, each at an MOI of 10, for 1 h at 31°C. At $t = 1$, the inoculum was replaced by 1.5 ml of DMEM–3% FCS, and the dishes were placed at 39.5°C. At $t = 3$, the medium was replaced by 500 μl of DMEM lacking both cysteine and methionine. After 1 h at 39.5°C, 15 μl of ^{35}S-Trans-label (10 μCi/μl) was added, and the cells were incubated for 15 min at 39.5°C. Cells from one dish of each virus were then lysed in 200 μl of MNT buffer (20 mM morpholineethanesulfonic acid, 30 mM Tris, 100 mM NaCl, 2.5 mM EDTA, 2 mM EGTA, 1% Triton X-100 [pH 5.8]). The medium of the other cells was replaced with 500 μl of DMEM containing 400 mM methionine and 400 mM cysteine, and incubation continued for 2 h at 39.5°C, followed by lysis in MNT buffer. After spinning down the nuclei, lysate supernatants were loaded on linear sucrose gradients (5 to 20% in MNT buffer with 0.1% Triton X-100) in SW55Ti tubes and centrifuged for 10 h at 45,000 rpm at 4°C. Fractions of 300 μl were collected from the gradients. Then 150 μl of each fraction was mixed with 10 μl of antispike MAb A2.3, incubated overnight at 4°C, precipitated in the presence of 50 μl of Pansorbin, and washed three times in RIPA buffer. The pellets were dissolved in 50 μl of Laemmli sample buffer and loaded on a 12.5% polyacrylamide gel.

Thermal inactivation. Thermal inactivation of virus grown at the permissive temperature was done as described previously (11). Viruses were incubated in 50 mM Tris-HCl (pH 6.5)–100 mM NaCl–1 mM EDTA–10% FCS at 39.5°C for different periods and then titered by plaque assay at 31°C.

RESULTS

Our studies into the role of the coronavirus MHV-A59 surface protein S in the replication cycle have to date been limited to systems in which a set of S-gene mutants was expressed transiently in the absence of the virus (1). Awaiting a reverse genetics approach to study S on virions, we decided to set up a system in which our mutant S proteins would complement viruses with defective S genes. To this end, we have reexamined a panel of *ts* mutants of MHV-A59, isolated in 1983 by Koolen et al. (12), and characterized two viruses unaffected in RNA synthesis at the nonpermissive temperature.

Virus titers. The two selected *ts* viruses, *ts*43 and *ts*379, and wt virus were grown on L cells at the nonpermissive temperature (39.5°C), harvested after 8 h, and subsequently plaqued on L cells at the permissive temperature (31°C). The virus titers were essentially the same as reported previously (12) and were at the nonpermissive temperature 1.2×10^4 (*ts*43), 2.8×10^5 (*ts*379), and 10^8 (wt) and at the permissive temperature 2×10^8 (*ts*43), 1.8×10^8 (*ts*379), and 5×10^8, (wt).

RNA synthesis. Next, the ability of each virus to produce RNA at the nonpermissive temperature was determined. L cells were infected with each virus at 31°C and shifted to 39.5°C after 1 h or kept at 31°C. RNA was then isolated at 19 h postinfection (p.i.) (31°C) or 7 h p.i. (39.5°C) and separated on an agarose gel. The RNA was visualized by hybridizing the dried gel to oligonucleotide 048, which recognizes the nested set of MHV-A59 RNAs. Figure 1 shows that there is no major difference in accumulation of RNAs between the permissive and nonpermissive temperatures for each virus, confirming the RNA^+ phenotype. Also, it can be concluded that there are no apparent differences in RNA accumulation between the three viruses, which is also true for earlier time points (data not shown).

Analysis of virion proteins. We first analyzed whether virus particles were being formed at the nonpermissive temperature. To find these particles, L cells were infected for 1 h with *ts*43, *ts*379, or wt virus at 31°C and then grown at either 31 or 39.5°C.

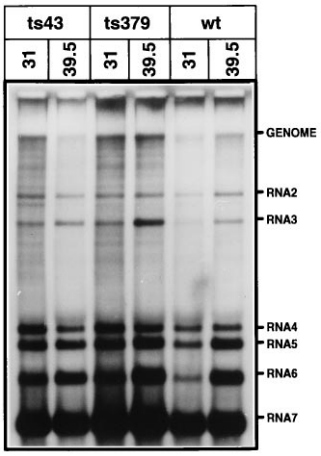

FIG. 1. Synthesis of viral RNA at the permissive and nonpermissive temperatures. RNA from L cells infected with MHV-A59 or *ts* mutant viruses was harvested 17 (31°C [permissive temperature]) or 8 (39.5°C [nonpermissive temperature]) h p.i. and separated on a polyacrylamide gel. Dried gels were hybridized to oligonucleotide 048, which binds to the 3′ end of positive-stranded viral RNA and recognizes the nested set of MHV RNA. At the top are shown the viruses used and the temperature at which they were allowed to replicate. The viral RNAs are indicated to the right.

At 11 h p.i. (31°C) or 3.5 h p.i. (39.5°C), the cells were labeled with [^{35}S]methionine for 3.5 h. Virus particles from the medium of the cells kept at 39.5°C were harvested directly, while the cells kept at 31°C were chased for 2 more h and then virus particles were harvested. Virus particles were purified on sucrose gradients. Figure 2 shows the result of polyacrylamide gel electrophoresis (PAGE) of virus directly loaded on a gel. At the permissive temperature, the three major structural proteins, the membrane protein M, the nucleocapsid protein N,

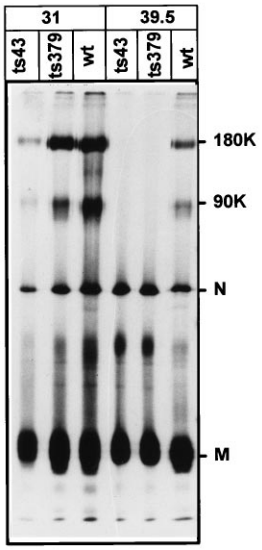

FIG. 2. Analysis of virion proteins of wt and *ts* mutant viruses grown at the permissive and nonpermissive temperatures. Viral proteins from L cells infected with wt and *ts* mutant viruses were labeled from 11.5 to 14.5 h p.i. (31°C), and virions were isolated from the medium at 16.5 h p.i. or labeled from 4 to 7 h p.i. (39.5°C) and then isolated from the medium at 7 h p.i. Virions were purified on linear sucrose gradients and directly subjected to PAGE. Viruses and the growth conditions are indicated at the top of the gel; protein species are indicated to the right.

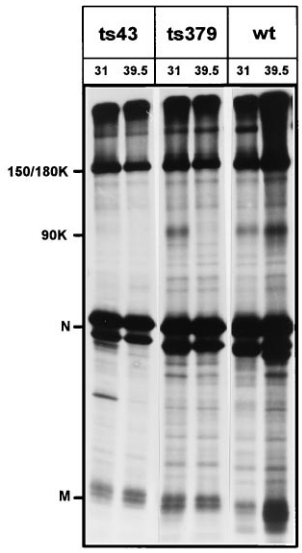

FIG. 3. Virus-specific proteins immunoprecipitated with polyclonal antiserum k134. Viral proteins from L cells infected with wt and *ts* mutant viruses were labeled from 10 to 13 (31°C) or 5 to 7 (39.5°C) h p.i., isolated, immunoprecipitated with polyclonal antibody k134, and subjected to PAGE. Viruses and the growth conditions are shown at the top of the gel; protein species are indicated to the left. 150/180K, 150K ER form of S protein/180K Golgi form of S protein; 90K, 90K cleavage product of 180K S protein; N, nucleocapsid protein; M, membrane protein.

and the surface protein S plus its 90K cleavage product, were easily detectable on wt and *ts* mutant virions, indicating normal virus production. In contrast, at the nonpermissive temperature, only wt virions contained these proteins. The *ts* mutants lacked both forms of the spike protein yet contained large amounts of the nucleocapsid and membrane proteins. This result shows that the *ts* mutants produce virus particles in quantities comparable to wt virus, but these particles lack spike proteins.

Synthesis of the structural proteins. To determine at what stage of *ts* virus replication the addition of S was blocked, we continued to study the synthesis and maturation of the viral structural proteins at the nonpermissive and permissive temperatures. Even when RNA synthesis is at a maximum, at 31°C (the permissive temperature), wt and *ts* mutant viruses each replicate at slightly different rates, which are approximately half the respective rates at 39.5°C (the nonpermissive temperature [reference 12 and data not shown]). This thwarts a direct comparison of the levels of the proteins produced by these viruses at the time point of analysis but does not prevent us from ascertaining whether the viral proteins are correctly matured. Virus-specific proteins from L cells infected with *ts*43, *ts*379, or wt MHV-A59 were labeled from 9 to 13 h p.i. (permissive temperature) or 4 to 7 h p.i. (nonpermissive temperature), and the lysates were immunoprecipitated.

Figure 3 shows the accumulation of the S, M, and N proteins immunoprecipitated with the polyclonal antibody k134. This antiserum will recognize all structural proteins of MHV-A59, including the 150K precursor of S180, but has a low affinity for the mature 180K form of the S protein. Expression of E is too low to be detectable by our antibodies. In contrast to the data presented by Koolen et al. (12), there was no significant difference in accumulation of the structural proteins either between the different viruses or between the permissive and nonpermissive temperatures in the lysates from infected cells. All three proteins accumulated to high levels. However, this anal-

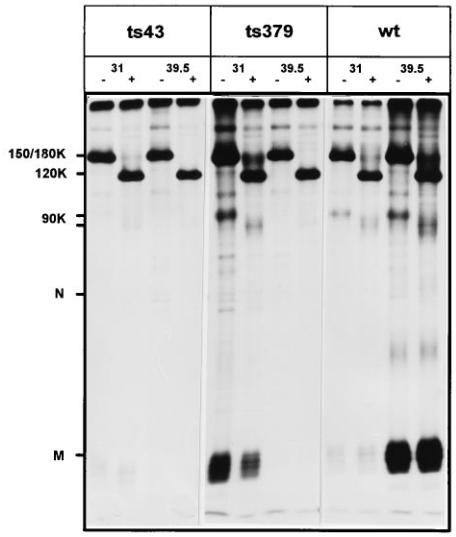

FIG. 4. Endo-H sensitivity of S-protein species. Viral proteins from L cells infected with wt and *ts* mutant viruses were labeled from 10 to 13 (31°C) or 5 to 7 (39.5°C) h p.i., isolated, immunoprecipitated with a mix of two MAbs directed at the S protein (A2.3 and A1.4), treated with endo-H, and subjected to PAGE. Viruses and the growth conditions are shown at the top of the gel; protein species are indicated to the left. −, without endo-H treatment; +, with endo-H treatment; 120K, 120K endo-H-deglycosylated form of S.

ysis did not allow us to determine which form of S was accumulating, although interestingly, for *ts*379 and *ts*43, the 90K protein was present at the permissive temperature only. This cleavage product of 180K S can be present only when the mature 180K S protein is present. The 90K protein was clearly detectable for wt virus at both temperatures. Several additional bands (those not indicated by the markers) which may represent breakdown products or aggregated proteins are detectable. They are often observed when the polyclonal serum is used for immunoprecipitation.

Maturation of the spike protein. In the previous experiment, accumulation of the spike protein was high at both the permissive and nonpermissive temperatures in the infected cell lysates. As mentioned, the polyclonal serum did not allow determination of the maturation species of the S protein. To study the fate of S in more detail, we used endo-H treatment, followed by immunoprecipitation with a mixture of anti-S MAbs A2.3 and A1.4, which are equally reactive to the different maturation forms of the S protein. Mature 180K spike proteins, on transport through the Golgi compartment, become endo-H resistant, whereas the ER, IC, and early Golgi 150K forms are endo-H sensitive. Since S150 and S180 are not resolved by PAGE, treatment with endo-H is necessary to detect the 180K form of S.

At the permissive temperature, all three viruses produced high levels of the 150K endo-H-sensitive form of the spike protein (Fig. 4). After endo-H treatment, most of the protein was shifted to the 120K deglycosylated form, and low amounts of 180K mature spike became detectable for each virus.

At the nonpermissive temperature, the differences in maturation of the S protein became evident. The 150K S and the 120K endo-H form of S were produced at the same levels as at the permissive temperature for each virus. However, only in the wt virus-infected cells was the mature 180K form of the spike protein detectable. No mature 180K S protein in the *ts* mutant virus-infected cells was detectable at the nonpermissive temperature. Also, the 90K cleavage product of 180K S was present for the *ts* mutant viruses at the permissive temperature only (only faintly visible for *ts*43). It was not detected at 39.5°C.

We conclude that maturation of the S protein of the *ts* mutant viruses occurred normally at the permissive temperature but was blocked at the nonpermissive temperature. The protein species represented by the bands that migrated slightly below where S120 migrates were most likely not S associated. They are commonly seen when this set of MAbs is used.

An important aspect of S maturation in infected cells is its association with M (23), which allows M to be coprecipitated with anti-S MAbs. The ability of M to interact with S per se was not influenced by temperature for the wt virus. Importantly, Fig. 4 clearly shows that for the *ts* mutant viruses, M coprecipitated with S only at the permissive temperature. However, there were clear differences in the amount of the M protein that coprecipitated, which may indicate different affinities of the S protein of the two *ts* mutant viruses for M.

Oligomerization of the spike protein. The difference in maturation of the S protein between wt and *ts* virus at the nonpermissive temperature observed in the previous experiment suggested a transport defect for the *ts* mutant spikes. Several studies have suggested that an important prerequisite for transport of membrane proteins is their correct folding and oligomerization in the ER (7, 9). Therefore, we went on to study the oligomerization of the spike proteins at the nonpermissive temperature.

Proteins from L cells infected with wt and *ts* mutant viruses were pulse-labeled for 15 min and isolated or chased for 2 hours and then isolated. The proteins were separated on linear sucrose gradients, and 15 fractions were collected and subjected to PAGE. Figure 5A shows that the S monomers, labeled during the short pulse, accumulated in fractions 5 to 7 for each virus. Two hours later, only the wt virus S proteins were oligomerized, as proven by a shift in migration from fractions 5 to 7 to the bottom of the gradient (Fig. 5B). Clearly, the *ts* mutant spike proteins remained in the same fractions of the gradient and thus did not form oligomers. The shifted S bands in the wt chase panel of Fig. 5B are double bands (only visible on a short exposure), representing 150K and 180K S. No 180K S was seen in any fraction of the *ts* mutant gradients. The chase experiment also revealed the presence of the 90K cleavage products of 180K S in the wt fractions. They were absent in the *ts* mutant virus fractions. Additional bands were observed above and below the position of 150/180K S. As can be seen from the wt chase panel of Fig. 5B, most of these band did not shift in the gradient. This may indicate that they represent folding intermediates or aggregates of misfolded S proteins.

Electron microscopy. To determine the morphology of virus particles produced at the nonpermissive temperature from wt and *ts* mutant virus-infected L cells, sucrose-purified virus preparations were studied by electron microscopy (Fig. 6). L cells infected with either *ts* mutant produced exclusively virus particles devoid of the characteristic spike projections, confirming the spikeless nature of the virus particles produced at 39.5°C. wt virus-infected L cells produced normal virus particles containing the spike corona, but these virions were in general highly pleomorphic, and up to 60% spikeless virus particles were seen in some wt preparations also (not shown).

Thermostability of *ts* virions. Conditionally lethal mutations affecting conformations of surface proteins often render these proteins thermolabile. We tested whether this was the case for *ts*43 and *ts*379 by incubating the viruses grown at the permissive temperature at 39.5°C for periods of up to 24 h. Surprisingly, *ts*43 was unaffected by this treatment. Its titers dropped only by a factor of 6, which was the same as for the control wt virus. However, *ts*379 appeared increasingly sensitive to high-tem-

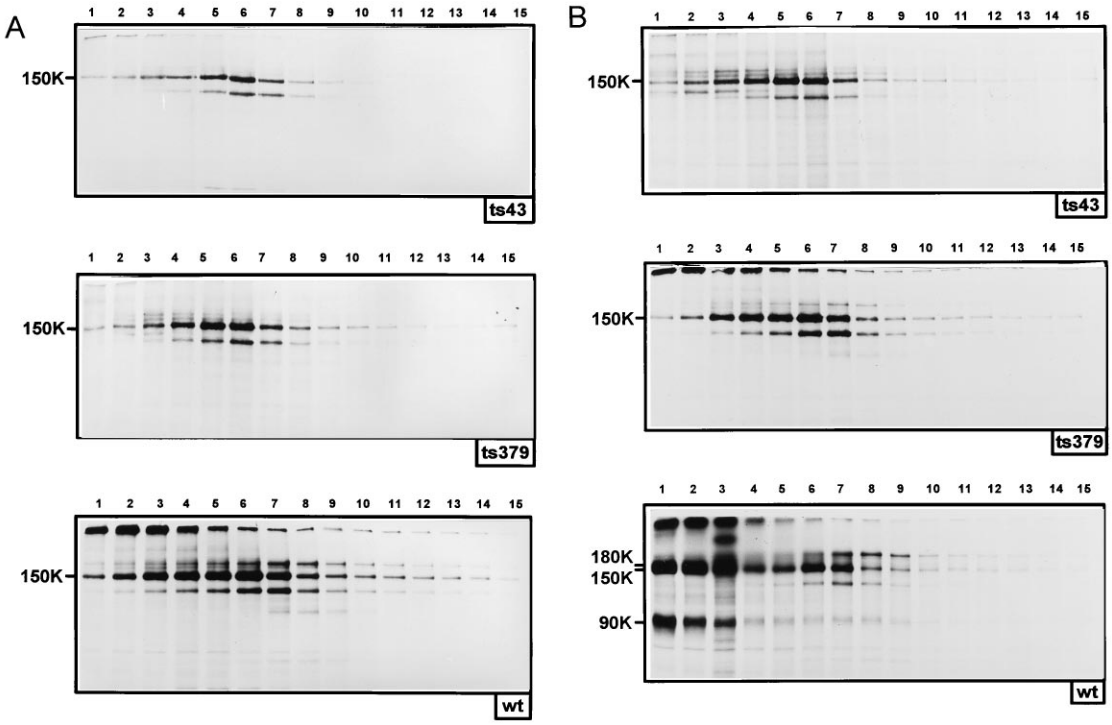

FIG. 5. Oligomerization of the S proteins of the wt and *ts* mutant viruses at the nonpermissive temperature. S proteins from L cells infected with wt and *ts* mutant viruses at 39.5°C were labeled for 15 min at 4 h p.i. and isolated (A) or additionally chased for 2 h and then isolated (B). Proteins were separated on a linear 5 to 20% sucrose gradient, and 15 fractions were immunoprecipitated with S-specific MAb A2.3 and subjected to PAGE (12.5% gel). Fraction numbers are indicated at the top (bottom of gradient = 1); protein species are indicated to the left.

perature incubation, resulting in at least a 5-log drop in infectivity after 6 h (Table 1). This drop in titer was not due to physiological conditions, because the titer of *ts*379 was unchanged when the virus was incubated at 0°C (data not shown). These data indicate that the *ts* lesion in the two mutant viruses is essentially of a different nature.

DISCUSSION

In this report, we describe two *ts* mutants of coronavirus MHV-A59, *ts*43 and *ts*379. The mutants are not impaired in RNA synthesis at the nonpermissive temperature (39.5°C), nor is the synthesis of the membrane and nucleocapsid protein

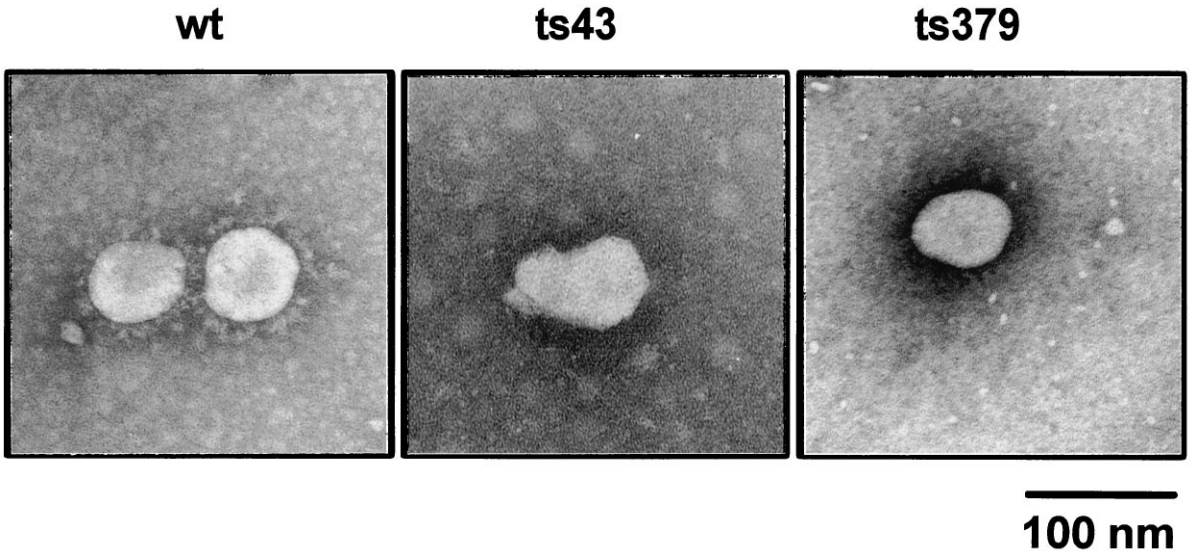

FIG. 6. Electron micrographs of wt and *ts* mutant virions produced at the nonpermissive temperature. Virus was grown on L cells at 39.5°C, harvested from the medium at 7 h p.i., and prepared for electron microscopy as described in Materials and Methods. The viruses are indicated at the top; the scale is shown at the bottom right.

TABLE 1. Sensitivity of *ts* mutant and wt viruses grown at the permissive temperature to incubation at the nonpermissive temperature[a]

Duration of incubation	*ts*43		*ts*379		wt	
	Titer[b]	R[c]	Titer	R	Titer	R
0 min	10^8	1	2×10^8	1	9×10^8	1
15 min	ND		2.6×10^7	0.13	10^8	0.12
60 min	ND		2.6×10^7	0.13	2.3×10^8	0.25
3 h	ND		2.6×10^5	0.013	10^8	0.12
6 h	ND		2×10^3	10^{-5}	10^8	0.12
24 h	1.5×10^7	0.15	$<6 \times 10^3$	7×10^{-5}	1.4×10^8	0.16

[a] MHV-A59 wt and *ts* mutants *ts*43 and *ts*379 were grown at 31°C and then incubated for different periods at 39.5°C.
[b] Plaque titer after the incubation period. ND, not determined.
[c] R, titer relative to that at $t = 0$ (no incubation), set at 1.

affected. However, *ts* mutant virions produced at the nonpermissive temperature are devoid of the surface protein, because the S proteins expressed by these viruses fail to exit from the ER.

The S protein of wt MHV-A59 is synthesized and cotranslationally N-glycosylated in the ER to a 150K protein. Folding and oligomerization take place with a half-time of approximately 1 h (21, 37), a process that likely occurs in the ER (6). Oligomerization of S is most likely required for the protein to be transported out of the ER, as it is for many other viral membrane proteins (7, 9, 27). Likewise, oligomerization itself is dependent on correct folding of the protein monomers (7). The membrane protein M forms a complex with S, probably with correctly folded S monomers before oligomerization occurs, since this interaction is detectable within 20 min of the onset of S synthesis (23). However, the M-S interaction is not required for S to be correctly oligomerized and transported to the cell surface. Studies in which S was transiently expressed by itself have indicated that its characteristics cannot be distinguished from those of S proteins produced in viral infections (1, 5, 37). The S-M oligomeric complexes are incorporated into virions budding into the IC (13). S is further glycosylated to the mature 180K form in the Golgi compartment. The *ts* mutant S proteins at the nonpermissive temperature are lacking from virions and are unable to mature to the 180K Golgi state. The earliest event in the S protein synthesis pathway that we could show to be different from wt is the oligomerization step. Since this step is dependent on correct monomer folding, the *ts* defect of the S proteins at the nonpermissive temperature is most likely incorrect folding of the spike monomers. In many cases, misfolded proteins form aggregates, destined to enter the degradation pathway (9). There is no clear evidence for aggregated S proteins, as no significant additional bands are present in the cell lysates analyzed at the nonpermissive temperature in Fig. 3 and 4 and no additional bands were observed in the *ts* gradient fractions from Fig. 5. However, it is possible that these aggregates are not recognized by the antibodies used in the immunoprecipitations under the experimental conditions. A better indication for correct folding of MHV-A59 spikes is their interaction with M (21), detected by coprecipitation of the membrane protein from cell lysates when anti-S MAbs are used. In the *ts* mutant-infected cells, no such coprecipitation is detectable at the nonpermissive temperature. Thus, we conclude that these S proteins are in a folding state that prevents interaction with M.

The fact that the two *ts* mutant viruses are differently sensitive to incubation at the nonpermissive temperature suggests that they contain different *ts* lesions. Preliminary data corroborate this observation in that revertant viruses are easily isolated for *ts*43 but not for *ts*379 (data not shown). Probably, the *ts* lesion in the S protein of *ts*43 allows it to acquire a stable structure, whereas that in *ts*379 does not. For other viruses, a lack of temperature sensitivity of *ts* virions could be correlated to protein maturation. Sindbis virus *ts* mutant *ts*23 has a defect in the E1 surface protein. When the *ts* E1 protein is allowed to form heterodimers with the second surface protein E2 at the permissive temperature, it is no longer *ts*, probably because the complex stabilizes its structure (3). In contrast, vesicular stomatitis virus (VSV) *ts*O45 virions are sensitive to incubation at an elevated temperature. At the permissive temperature, the VSV surface protein G on *ts*O45 is folded and oligomerized with wt kinetics and reportedly interacts with the matrix protein to form stable hetero-oligomers yet remains heat sensitive (14, 16). It will be interesting to learn whether the difference in thermolability between *ts*43 and *ts*379 can be linked to putative stabilizing interactions with other viral proteins, particularly the membrane protein. There is a difference in affinity for M between the two *ts* mutant S proteins, as can be judged from the coprecipitation data. Experiments are under way in our lab to study *ts* S-M interactions in more detail.

Recently, a *ts* mutant of MHV-A59, *ts*18, with a mutation in the spike gene that was sensitive to incubation at 40°C and that also produced spikeless virus particles was described (26). The surface protein was not characterized in detail; therefore, its phenotype cannot be linked to specific maturation defects. Fu and Baric (8) described several MHV-A59 *ts* mutants with mutations in the spike gene. For these mutants, only the location of the mutation was mapped; the S phenotypes were not investigated.

The *ts* mutant viruses that we have characterized again show that coronaviruses can produce particles without spike proteins, as was observed previously (10, 29). Similar findings have recently been reported for rabies virus (19). Recent experiments have shown that coronavirus-like particles can be produced from cells in which the viral structural proteins are expressed in the absence of the spike protein gene (2, 36). These findings, together with our observation that the *ts* S protein is retained in the ER, argue against the possibility that a membrane-embedded domain of *ts* S is left behind after an unspecified degradation process in the ER, to be incorporated in virions, as was shown for the surface protein of VSV *ts*O45 (20). Our analysis of virion proteins showed that there was no apparent difference in particle production at the nonpermissive temperature between wt and *ts* viruses when judged by the levels of the membrane and nucleocapsid proteins. This finding indicates that S plays no significant stimulatory role in virion production.

We are currently cloning, expressing, and sequencing both *ts* mutant S genes to locate the *ts* mutations. We plan to use the *ts* mutant viruses in complementation studies with our panel of mutant S genes (1). Such an approach has been used successfully for other viruses, such as VSV (38, 39) and influenza virus (30), and will produce new insights in the role of the spike protein in the coronavirus life cycle.

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