The coronavirus replicase-transcriptase complex is an assembly of viral and cellular proteins that mediate the synthesis of genome and subgenome-sized mRNAs in the virus-infected cell. Here, we report a genetic and functional analysis of 19 temperature-sensitive (ts) mutants of Murine hepatitis virus MHV-A59 that are unable to synthesize viral RNA when the infection is initiated and maintained at the non-permissive temperature. Both classical and biochemical complementation analysis leads us to predict that the majority of MHV-A59 ORF1a replicase gene products (non-structural proteins nsp1–nsp11) form a single complementation group (cistron1) while the replicase gene products encoded in ORF1b (non-structural proteins nsp12–nsp16) are able to function in trans and comprise at least three, and possibly five, further complementation groups (cistrons II–VI). Also, we have identified mutations in the non-structural proteins nsp 4, nsp5, nsp10, nsp12, nsp14, and nsp16 that are responsible for the ts phenotype of eight MHV-A59 mutants, which has led us to conclude that these proteins are essential for the assembly of a functional replicase-transcriptase complex. Finally, our analysis of viral RNA synthesis in ts mutant virus-infected cells allows us to discriminate three phenotypes with regard to the inability of specific mutants to synthesize viral RNA at the non-permissive temperature. Mutant LA ts6 appeared to be defective in continuing negative-strand synthesis, mutant Alb ts16 appeared to form negative strands but these were not utilized for positive-strand RNA synthesis, and mutant Alb ts22 was defective in the elongation of both positive- and negative-strand RNA. On the basis of these results, we propose a model that describes a pathway for viral RNA synthesis in MHV-A59-infected cells. Further biochemical analysis of these mutants should allow us to identify intermediates in this pathway and elucidate the precise function(s) of the viral replicase proteins involved.

Introduction

Coronaviruses are positive-strand, enveloped RNA viruses that infect vertebrates and are associated mainly with respiratory and enteric disease. They have long been recognized as important pathogens of livestock and companion animals, and they are a common cause of respiratory tract infections in humans [1–3]. More recently, a coronavirus has been identified as the causative agent of SARS, a form of atypical pneumonia in humans with a case fatality ratio of approximately 10% [4]. Clearly, there is an urgent need to develop new strategies to prevent or control coronavirus infections, and understanding the biology, replication, and pathogenesis of these viruses is an essential part of this process. Murine hepatitis virus, strain A59 (MHV-A59), is a group II coronavirus with a genome of approximately 31,400 nucleotides. The genomic RNA encodes the structural proteins of the virus, non-structural proteins involved in viral RNA synthesis (the nsp or replicase proteins), and proteins that are non-essential for replication in cell culture but appear to confer a selective advantage in vivo (accessory proteins) [1]. In the MHV-A59-infected cell, the expression of the replicase protein genes is mediated by translation of the genomic RNA, and the expression of the structural protein genes is mediated by the translation of a set of 3′-coterminal subgenomic mRNAs. The subgenomic mRNAs are produced by a unique mechanism that involves discontinuous transcription during negative-strand RNA synthesis [5–7]. The organization and expression of the MHV-A59 genome are illustrated in Figure 1.

The 5′ proximal open reading frames (ORF) of MHV-A59 genomic RNA (ORF1a and ORF1b) are translated to produce two large polyproteins, ppla and pp1ab, with calculated

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molecular masses of 496.6 and 802.8 kilodaltons, respectively. Translation of the larger pp1ab involves programmed (−1) ribosomal frameshifting [8]. During or after synthesis, these polypeptides are extensively processed by three virus-encoded proteinases to produce a membrane-bound replicase-transcriptase complex [9]. Cleavage of the replicase polyproteins is predicted to result in 16 end-products; nsp1–nsp11 encoded in ORF1a and nsp12–16 encoded in ORF1b [10]. These proteins have been shown, or are predicted to have multiple enzymatic functions, including papain-like proteases (nsp3), adenosine diphosphate-ribose 1’-phosphatase (nsp3), 3C-like cysteine proteinase (nsp5), RNA-dependent RNA polymerase (nsp12), superfamily 1 helicase (nsp13), exonuclease (nsp14), endoribonuclease (nsp15), and S-adenosylmethionine-dependent 2’-O-methyl transferase (nsp16) [11–20]. The crystallographic structures of SARS coronavirus nsp5 and nsp9 have been determined and are likely to be similar for MHV-A59 [21–23].

In the course of an infectious cycle, the MHV-A59 replicase-transcriptase complex amplifies the genomic RNA and synthesizes subgenomic mRNAs. Amplification of the genomic RNA involves full-length negative-strand templates, and the synthesis of subgenomic mRNA involves subgenome-length negative-strand templates [24,25]. The structures engaged in the replication and transcription of positive-strand MHV-A59 RNA have been characterized [26]. Approximately 70% of the replicating and transcribing structures that accumulate in infected cells are multi-stranded intermediates (replicative and transcriptive intermediate RNA, RI/TI RNA) and 30% are found in structures with only one or very few nascent strands (native replicative and transcriptive forms, RF/TF RNA). Although the structures engaged in negative-strand RNA synthesis have not yet been characterized, it is known that MHV negative-strand templates are unstable and turn over during viral replication [27].

The cis-acting RNA elements involved in the different phases of MHV RNA synthesis have been studied quite extensively. It has been shown that 5’- and 3’-UTR, as well as...
5'-UTR-adjacent regions of the genome are required for MHV replication and transcription [28,29]. Also, studies on MHV, and other nidoviruses, have shown the critical role of the so-called transcription-regulating sequence (TRS) element in the discontinuous phase of the transcription process [7,30–33]. These data show that the stability of the leader-TRS/body-TRS duplex, which forms during the discontinuous extension phase of negative-strand template synthesis, is an important determinant of subgenomic mRNA abundance. However, it is also evident from these studies that the regions flanking the TRS elements have a profound effect on the amounts of subgenomic mRNAs that are produced. In the context of the discontinuous-extension model [5], this is explained as different degrees of “attenuation” at each of the TRS elements during negative-strand synthesis.

In contrast, there is still very little known about the structure, functions, and interactions of viral and cellular proteins in the replicase-transcriptase complex as it is engaged in different modes of RNA synthesis. As mentioned above, bioinformatic and biochemical studies have identified a number of (putative) enzymatic activities associated with individual coronavirus replicase proteins, and a number of cellular proteins have also been implicated as components of the MHV replicase-transcriptase complex [34–36]. However, the essential nature of some of these cellular proteins has been questioned [37], and further work is needed to determine the exact protein composition of the coronavirus replicase-transcriptase complex and how the composition is altered, or how the proteins are modified to regulate the different activities of the complex.

In order to address these sorts of questions, we have embarked upon a detailed analysis of temperature-sensitive (ts) mutants of MHV-A59 that are unable to synthesize viral RNA when the infection is initiated and maintained at the non-permissive temperature. The essential feature of these mutants is that they are likely to be defective in different aspects of viral RNA synthesis and a detailed characterization of their genotype and phenotype should provide insights into the mechanisms of RNA synthesis, the functions of individual viral replicase proteins, and the protein-RNA and protein–protein interactions that regulate the activity of the replicase–transcriptase complex. These conditional-lethal mutants may also be used in a cis–trans test to define the number of complementation groups, or cistrons, that contribute to a specific phenotype. This sort of analysis can also provide valuable insight into the possible pathways that polyproteins must travel to assume functional configurations and has been used with success for other RNA viruses [38].

The MHV-A59 mutants that we have studied have been produced in a number of laboratories over a period of 20 years [39–41]. They have been selected to have a low efficiency of plaque formation at the non-permissive temperature compared with the permissive temperature and hence a reversion frequency indicative of single point mutations. In this study, we describe a complementation analysis, and by sequence analysis of both ts virus and revertants, we identify the causal mutation for eight of these mutants. We also describe a more detailed phenotype for selected mutants and suggest a model that describes the different modes of RNA synthesis during coronavirus replication and transcription.

Results

Characterization of ts Mutants and Revertants

Table S1 lists the ts mutants of MHV-A59 used in our collection. All the ts mutants failed to form plaques or synthesize viral RNA when infection was initiated and maintained at the non-permissive temperature. While many mutants failed to form plaques at 37 °C, other mutants formed plaques at 37 °C and were considered leaky. This included Alb ts22 that produced pin-prick-sized plaques after 2 d at 37 °C (compared with the wild-type [wt] A59 virus, which produced uniform plaques of 4–5 mm in diameter) and Wu ts18, Wu ts36, and Wu ts38, which produced smaller than wt plaques at 37 °C. However, even for these mutants, the ts defects responsible for their RNA-negative phenotype appeared to be caused by a single point mutation because each ts mutant possessed a characteristic low reversion frequency between $10^{-4}$ and $10^{-8}$ per average base [42]. The virus produced at 37 °C by Alb ts22, Wu ts18, Wu ts36, and Wu ts38 was also ts, i.e., the efficiency of plating (EOP) was less than $10^{-4}$.

For most mutants, the revertant virus obtained from plaques formed at the non-permissive temperature had properties identical to wt MHV-A59. One exception was Alb ts17, which produced equal numbers of revertant viruses causing A59-sized plaques and revertant viruses with noticeably smaller plaques (Figure S1). We isolated revertant viruses from a large (A59-sized) plaque (Alb 17R L) and a small plaque (Alb 17R R) for sequence analysis (see below). Some of the ts mutants did not produce revertant viruses (e.g., LA ts3, Alb ts19) or produced revertant viruses that were markedly different from the parental MHV-A59 virus.

Complementation Analysis

We began our complementation analyses using Alb ts16, LA ts6, and Alb ts22 because they each had a distinct ts viral RNA synthesis phenotype (see below). Cells were singly infected or doubly infected with two ts mutants and the cells and medium were harvested after the completion of a single round of replication, i.e., 8 h post-infection (hpi) at 40 °C. We also confirmed that if infection with a ts virus alone was allowed to proceed for up to 2 h at 30 °C, and then the culture shifted to 40 °C and the virus harvested at 12 hpi, the titer we obtained was low ($-10^4$ plaque-forming units [pfu]/ml). Thus, this protocol prevented the production of revertant virus by a second round of replication. Complementation was measured by determining the complementation index (CI) as described in Materials and Methods. By definition, if the mutations are in the same cistron, the viruses will not complement each other. On the other hand, if the mutations are in different cistrons, the mutants will complement each other and progeny ts virus will be recovered.

The results of six individual crosses between Alb ts16 and LA ts6 are shown in Table 1. All of these crosses failed to show complementation. The average CI value was 0.5 (0.5 ± 0.18 SD), which is the theoretical value for two mutants with mutations in the same cistron [43]. This CI value was obtained using only the titers determined at 30 °C and was not corrected for the presence of revertants (or recombinants) as was done by others [39,44]. We found it unnecessary to make this correction because it did not significantly change the CI value (at most a decrease of one tenth) and whether or not the
Table 1. Genetic Complementation Analysis of MHV-A59 ts Mutants

| Cross       | CI     | MOI (pfu/Cell)* |
|-------------|--------|-----------------|
| Alb ts16 × LA ts6 | 0.35   | 20 ± 20         |
| Alb ts16 × LA ts6 | 0.54   | 20 ± 20         |
| Alb ts16 × LA ts6 | 0.65   | 20 ± 20         |
| Alb ts16 × LA ts6 | 0.24   | 20 ± 20         |
| Alb ts16 × LA ts6 | 0.51   | 20 ± 20         |
| Alb ts16 × LA ts6 | 0.71   | 100 ± 100       |
| LA ts6 × Alb ts22 | 11     | 20 ± 20         |
| LA ts6 × Alb ts22 | 18     | 20 ± 20         |
| LA ts6 × Alb ts22 | 110    | 100 ± 100       |
| Alb ts16 × Alb ts22 | 121    | 20 ± 20         |
| Alb ts16 × Alb ts22 | 121    | 20 ± 20         |
| Alb ts16 × Alb ts22 | 107    | 100 ± 100       |
| Alb ts6 × Alb ts16 | 0.23   | 100 ± 100       |
| Alb ts6 × LA ts6 | 1.7    | 100 ± 100       |
| Alb ts6 × Alb ts22 | 694    | 20 ± 20         |
| Alb ts6 × Alb ts22 | 108    | 100 ± 100       |
| Alb ts6 × Wu ts18 | 141    | 20 ± 20         |
| Alb ts6 × Wu ts36 | 183    | 20 ± 20         |
| Alb ts6 × Wu ts38 | 1,875  | 20 ± 20         |
| Alb ts22 × Wu ts18 | 185    | 20 ± 20         |
| Alb ts22 × Wu ts36 | 240    | 20 ± 20         |
| Alb ts22 × Wu ts38 | 1,300  | 20 ± 20         |

*Cells infected with either 25 pfu or 100 pfu of each ts virus per cell.

This table shows the results of genetic complementation analysis of MHV-A59 ts mutants. The CI (complementation index) and MOI (multiplicity of infection) values are reported for various crosses of ts mutants. The results indicate that some ts mutants failed to complement each other, while others did, thus defining different complementation groups.

Figure 2. Biochemical Complementation Analysis of Selected MHV-A59 ts Mutants

Cells were mock-infected or infected with MHV-A59, one of the ts mutants, or with a mixture of two ts mutants. The cells were incubated at 40 °C in medium containing dactinomycin and 3H-uridine and, at 8 hpi, 3H-uridine incorporation into trichloroacetic acid-precipitated RNA was determined. Cells were infected with: M, mock-infected; A59, MHV-A59; A6, Alb ts6; A16, Alb ts16; A22, Alb ts22; A17, Alb ts17; L6, LA ts6; W18, Wu ts18; W36, Wu ts36; W38, Wu ts38; A6x16, Alb ts6 and Alb ts16; A6xL6, Alb ts6 and LA ts6; A6x22, Alb ts6 and Alb ts22; A16xL6, Alb ts16 and LA ts6; A16x22, Alb ts16 and Alb ts22; A17xL6, Alb ts17 and LA ts6; A17x22, Alb ts17 and Alb ts22; A17xW18, Alb ts17 and Wu ts18; A17xW36, Alb ts17 and Wu ts36; A17xW38, Alb ts17 and Wu ts38; A22xL6, Alb ts22 and LA ts6; A22xW18, Alb ts22 and Wu ts18; A22xW36, Alb ts22 and Wu ts36; A22xW38, Alb ts22 and Wu ts38; W18xW36, Wu ts18 and Wu ts36; W18xW38, Wu ts18 and Wu ts38; W36xW36, Wu ts36 and Wu ts36.

This finding provides the means to develop a more convenient and more rapid method of determining complementation for MHV-A59 ts mutants. We reasoned that because recombination appeared to be driven by complementation, biochemical complementation (i.e., viral RNA synthesis) might be detected in cells co-infected with complementing ts mutants, but not in cells infected with ts mutants in the same complementation group. We devised such an assay. Cells were infected at the permissive temperature and were then re-fed with medium prewarmed to the non-permissive temperature and containing dactinomycin to inhibit DNA-dependent RNA synthesis and 3H-uridine to label viral RNA. The infected cells were incubated until 7–8 hpi at 39 °C to 40 °C or 8–12 hpi at 30 °C, and RNA synthesis was measured by the incorporation of 3H-uridine into acid-precipitable material. Figure 2A shows the results of single and double infection with the Alb ts6, Alb ts16, Alb ts22, and LA ts6 mutants. The data show that at 40 °C, the mutants Alb ts6, Alb ts16, and LA ts6 were not able to rescue the RNA-negative phenotype of each other and thus, the three mutants were in the same complementation group. In contrast, Alb
ts22 was able to rescue the RNA-negative phenotype of Alb ts6, Alb ts16, and LA ts6, and thus, was the sole member of a separate complementation group. This result is identical to that obtained using classical complementation assays and served to validate the new method. The assay was as specific as classic genetic complementation, which measures progeny incorporation was less than that obtained from mock-infected cells, and thereby was statistically indistinguishable and was combined to give an average background incorporation that was not determined.

Using this assay, we were able not only to confirm the prediction of at least three complementation groups that we have tentatively named cistrons I, II, IV, and VI based on the locations identified for their causal point mutations (see below). This numbering scheme leaves open the possibility of finding two additional complementation groups (cistrons III and V) in the future that would represent gene products of ORF1b (see below).

Identification of Mutations Responsible for the ts Mutant Phenotype

The entire coding region of the replicase genes (ORF1a and ORF1b) was sequenced for each of eight ts mutant/revertant pairs. In each case, a single nucleotide change was identified as the mutation responsible for the ts mutant phenotype. Using the numbering that we have assigned to the infectious cDNA clone of the MHV-A59 genome [45] (GenBank accession number AY700211), the nucleotide changes compared to wt MHV-A59 were identified as: Alb ts6, A36609C; Alb ts16, U19364C; Alb ts18, C13560G; Alb ts22, A69180G; Alb ts17, G19288A; Wu ts38, U16838C; Wu ts18, C29880U; Wu ts36, U21904C (Figure 3A). We also identified a number of nucleotide differences between mutants isolated in different laboratories, but in no case did they correlate with the ts phenotype. With the exception of the Alb 17R, revertant, all of the revertants we isolated were true, i.e., they were genetically and phenotypically identical to the wt MHV-A59. The Alb 17R revertant was a pseudorevertant in that the nucleotide at position 19288 had reverted from A to C, which resulted in a substitution of Tyr with Arg. This radical substitution was reflected in a small plaque phenotype (Figure 3B).

All of the nucleotide changes responsible for the ts mutant phenotype were non-synonymous mutations. The amino acid substitutions are shown in Figure 3B. Conservative substitutions were identified in nsp5 and nsp10 of the Alb ts6 and LA ts6 mutants, respectively. Moderately conservative substitutions were identified in nsp4 and nsp12 of the Alb ts6 and Alb ts22 mutants, respectively. And radical substitutions were identified in nsp4 of the Alb ts17 and Wu ts38 mutant, as well as nsp16 of the Wu ts18 and Wu ts36 mutants [46]. A comparison of the predicted replicase protein sequences from different coronaviruses showed that there was, by large, conservation of the amino acids that were substituted in the proteins with a ts phenotype. For example, the Gln465 residue of nsp10, the His968 residue of nsp12, and the Cys988 residue of nsp14 appear to be well conserved in Group I, II (including SARS-CoV), and III coronaviruses. In contrast, the Asn528 residue of nsp4 is only found in MHV strains, although in the majority of other coronaviruses, it is substituted by an aspartic acid. Finally, it is possible, with different degrees of confidence, to predict the structural environment in which the residues in question are found. On the one hand, it is highly likely that the Phe210 residue of nsp5 is located in an extended area that connects the α-helices B and C in the carboxy-terminal domain III of nsp5, the 3C-like proteinase. This conclusion is based upon the similarity in the sequences of coronavirus nsp5 proteins and the crystallographic

### Table 2. Biochemical Complementation Analysis of MHV-A59 ts Mutants

| Mutants | Complementation Groups |
|---------|------------------------|
|         | I         | II         | IV         | VI         |
| Alb ts16 | —         | 0.2        | 39         | 50         | ND         |
| Alb ts2  | 0.9       | 0.1        | 56         | 52         | 53         |
| Alb ts6  | 0.1       | 0.1        | 41         | ND         | ND         |
| Alb ts8  | 0.4       | <0         | 31         | 50         | 50         |
| Alb ts9  | 0.2       | 0.3        | 49         | 52         | 54         |
| Alb ts19 | 0.1       | <0         | 30         | 37         | 17         |
| Ut ts88  | 0.3       | <0         | 11         | 31         | 13         |
| Ut ts329 | 0.4       | <0         | 45         | 57         | 30         |
| LA ts3   | 0.1       | 0.3        | 22         | 39         | 14         |
| LA ts6   | <0        | —          | 9          | 3          | ND         |
| LA ts9   | 0.1       | <0         | 9          | 37         | 14         |
| NC ts2   | 0.1       | <0         | 15         | 25         | 15         |
| NC ts3   | <0        | 0.23       | 6          | 21         | 6          |
| Alb ts22 | ND        | ND         | —          | 38         | 6          |
| Alb ts17 | 77        | 77         | 47         | —          | 58         |
| Wu ts38  | ND        | ND         | 15         | 0.3        | 40         |
| Wu ts18  | 39        | ND         | 6.6        | 42         | —          |
| Wu ts36  | ND        | ND         | 7.2        | 37         | <0         |
| Ut ts145 | 6         | 6          | 7          | 35         | 0.1        |

The numbers represent the percent of the incorporation found in the MHV-A59 infected cells.

*H*-uridine incorporation into trichloroacetic acid-precipitated RNA in the mock- and singly ts mutant-infected cells was statistically indistinguishable and was combined to give an average background incorporation that was subtracted from the MHV-A59 and the doubly infected samples.

ND, not determined.

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| ts88 and ts329; LA ts3 and ts9; and NC ts2 and ts3) to the same complementation group as Alb ts6, Alb ts16, and LA ts6 and, it was possible to assign mutant Ut ts145 to the same complementation group as Wu ts18 and Wu ts36. Thus, it was possible to assign the entire collection of 19 RNA-negative ts mutants of MHV to one of four complementation groups, which we have tentatively named cistrons I, II, IV, and VI based on the locations identified for their causal point mutations (see below). This numbering scheme leaves open the possibility of finding two additional complementation groups (cistrons III and V) in the future that would represent gene products of ORF1b (see below).
structures that have been solved for the transmissible gastroenteritis virus (TGEV), SARS-CoV, and HCoV-229E nsp5 proteins [23,47,48]. On the other hand, programs that predict protein secondary structure [49] indicate that the Gln
\[65\]
residue of nsp10, the His
\[868\]
residue of nsp12, and the Cys
\[408\]
residue of nsp14 are located in disordered loop structures, while the Asn
\[258\]
residue of nsp4 and the Leu
\[153\]
residue of nsp16 are involved in \(\alpha\)-helices. Obviously, more definitive structural data will be needed to confirm these predictions.

Phenotypes of the MHV-A59 ts Mutants

We focused our phenotypic analysis on the eight MHV-A59 ts mutants that had been genotyped and began by measuring total viral RNA synthesis in infected cells prior to and following shift from the permissive to the non-permissive temperature. This analysis was done after 8 h of incubation at \(30^\circ\)C, a time at which the replicase-transcriptase complex produces mainly (\(\approx 90\%\)) positive-strand RNA, and \(-20\%\) of the maximum rate of RNA synthesis has been reached. Mutant virus-infected cells were shifted to \(40^\circ\)C at 8 hpi and a duplicate set was left at \(30^\circ\)C. Both sets of cultures were labeled for 1 h with \(^3\)H-uridine in the presence of cycloheximide (CH) to monitor the replicase-transcriptase activity at the time of shift. The results are shown in Figure 4. In MHV-A59 infected cells, the amount of \(^3\)H-uridine incorporation doubled, as expected, when the temperature was increased by \(10^\circ\)C. The group I mutants had about the same level of viral RNA synthesis at both temperatures, while in the group II, IV, and VI mutant-infected cells, viral RNA synthesis diminished by \(50\%\) or more in the hour following temperature shift. We interpret

![Figure 3. Genotypic Analysis of Selected MHV-A59 ts Mutants](image)

(A) The positions of mutations responsible for the ts phenotype of selected MHV-A59 mutants are illustrated in relation to the non-structural proteins (nsp1–16) produced by proteolytic processing of the ORF1a/ORF1b polyprotein, pp1ab. Nucleotide changes are numbered according to the sequence of the infectious cDNA clone of MHV-A59.

(B) The amino acid substitutions responsible for the mutant and revertant phenotypes are listed together with the mutated protein and the cistron to which each mutant has been assigned. The amino acids are numbered from the amino-terminus to the carboxyl-terminus of each of the non-structural proteins.

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![Figure 4. RNA Synthesis Phenotype of MHV-A59 ts Mutants](image)

RNA synthesis was determined using a 1 h pulse label with \(^3\)H-uridine in the presence of dactinomycin and cycloheximide, given to wt MHV-A59 and ts mutant virus-infected cells at 8 hpi with or without shifting from the permissive to the non-permissive temperature. The amount of incorporated \(^3\)H-uridine at \(40^\circ\)C was divided by that at \(30^\circ\)C and 1.0 was subtracted. The results represent the average of five separate experiments. A value of zero means the incorporation at the two temperatures was the same.

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Figure 5. RNA Synthesis Phenotype of the Alb ts22 Mutant

RNA synthesis was determined (A) using 1 h pulse labels with H-uridine in the presence of actinomycin, given to MHV-A59-, Alb ts22-, and Alb 22R-infected cells 1–6 hpi at 40 °C or 5–14 hpi at 30 °C; ○, 40 °C, Alb ts22; □, 30 °C, wt MHV-A59; △, 30 °C, Alb 22R; ◊, 30 °C, Alb ts22, or (B) using 30 min pulse labels with H-uridine in the presence of actinomycin, given to MHV-A59-, Alb ts22-, and Alb 22R-infected cells after shift from the permissive to the non-permissive temperature at 13 hpi; ▲, wt MHV-A59; ◂, Alb 22R; ●, Alb ts22.

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this to mean that mutations in replicase proteins encoded in ORF1a appeared to confer temperature-sensitivity to the viral replicase-transcriptase complex, but once it had formed at 30 °C, its positive-strand synthetic activity was relatively resistant to higher temperature. In contrast, mutations in ORF1b-encoded proteins, namely nspl2, nspl4, and nspl6 appeared to affect the positive-strand synthetic activity of already-formed replicase-transcriptase complexes. We then went on to analyze the phenotypes of three ts mutants in more detail.

Alb ts22. The phenotype described above for group II, IV, and VI mutants would be consistent with a defect in any stage of positive-strand RNA synthesis. In the case of mutant Alb ts22, however, we have shown that the ts lesion is located in nspl2, the viral RNA-dependent RNA polymerase subunit. This suggested to us that the Alb ts22 might be defective in the elongation phase of RNA synthesis. To analyze the phenotype of Alb ts22 in more detail, RNA synthesis in Alb ts22-infected cells was determined using 1 h pulse labels with H-uridine in the presence of actinomycin, given between 1–6 hpi at 40 °C or between 5–14 hpi at 30 °C (Figure 5A). At 40 °C, Alb ts22-infected cells incorporated only low levels of H-uridine, as expected for an RNA-negative ts mutant. In contrast, cells infected with wt MHV-A59 or with Alb 22R (a revertant of Alb ts22) made RNA at high rates and at identical times. At 30 °C, Alb ts22 was defective in viral RNA synthesis and never reached the levels of viral RNA synthesis shown by wt MHV-A59 or Alb 22R. These results are consistent with our finding that, at 30 °C, the plaques formed by Alb ts22 were smaller that those formed by wt MHV-A59. Analysis by gel electrophoresis of the species of positive-strand RNA made in Alb ts22-infected cells at 30 °C showed the typical pattern of seven RNAs (genome and six subgenomic mRNAs), although the six subgenomic mRNAs were reduced equally in amount relative to the genome RNA when compared to Alb 22R infected cells (unpublished data). We conclude that Alb ts22 not only produced less overall RNA compared to wt MHV-A59 and Alb 22R, even at the permissive temperature, but also under-produced all of the subgenomic mRNA species relative to the genome RNA.

We also examined the ability of Alb ts22-infected cells to continue viral RNA synthesis after shift from 30 °C to 40 °C at 13 hpi (Figure 5B). This allowed us to follow the activity at 40 °C of the viral RNA-dependent RNA polymerase that was made and assembled at 30 °C. At this time, Alb ts22 RNA synthesis was at its maximum rate and RNA synthesis by wt MHV-A59 and Alb 22R was declining. The results show that a shift to 40 °C led to the rapid loss of RNA synthesis by Alb ts22 but not by wt MHV-A59 or Alb 22R. This result is consistent with a failure of the viral RNA-dependent RNA polymerase to continue transcription at the non-permissive temperature. We concluded Alb ts22 had a ts defect in elongation, although we do not know if elongation is directly affected or if the amino acid change in nspl2 affects its interaction with an as yet unknown, but essential protein. We have also shown that, as expected, Alb ts22 is unable to synthesize negative-strand RNA at the non-permissive temperature (unpublished data).

Alb ts16 and LA ts6. Although both Alb ts16 and LA ts6 are unable to synthesize viral RNA when the infection is initiated and maintained at the non-permissive temperature, the data shown in Figure 4 suggests that they are not significantly impaired in their ability to synthesize positive-strand RNA at this temperature. This conclusion is strengthened by the results shown in Figure 6A, which demonstrate the kinetics of overall viral RNA synthesis in Alb ts16 and LA ts6 virus-infected cells after shifting the incubation temperature from 30 °C to 40 °C at 8 hpi. With wt MHV-A59, viral RNA synthesis increased rapidly within the first 60 min after temperature shift, consistent with the synthesis of both additional negative-strand templates and their nascent positive-strand product. The addition of CH at the time of shift resulted in a constant rate of viral RNA synthesis for at least 1 h. As we know that negative-strand synthesis in MHV-A59-infected cells is short-lived and stops within 30 min of the inhibition of protein synthesis [24], we deduce that the addition of CH prevented the synthesis of new viral proteins, which in turn prevented the formation of additional replicase-transcriptase activity and negative-strand templates.

In cells infected with complementation group I ts mutants Alb ts16 and LA ts6, viral RNA synthesis continued at 40 °C at the level ongoing at the time of temperature shift (Figure 6A). This meant that the replicase-transcriptase complexes assembled at 30 °C continued to function at 40 °C in the synthesis of positive-strand RNA. However, unlike A59-infected cells, the group I mutants did not increase their rates of RNA synthesis after shift to non-permissive temperature, indicating that no new active complexes were formed. This phenotype resembled that seen with MHV-A59-infected cells short-lived and stops within 30 min of the inhibition of protein synthesis [24], we deduce that the addition of CH prevented the synthesis of new viral proteins, which in turn prevented the formation of additional replicase-transcriptase activity and negative-strand templates.

At least two possibilities could account for a failure of group I ts mutants to form fully competent replicase-transcriptase complexes at the non-permissive temperature. Either no new negative-strand templates were made, i.e., a defect in negative-strand synthesis, or, if they were made, they could not be used as templates for positive-strand synthesis.
The latter phenotype has been observed for certain alphavirus mutants [50], which were called conversion-defective mutants. To distinguish between these two possibilities, it is necessary to shift the ts mutant-infected cells to the non-permissive temperature and determine their ability to continue negative-strand RNA synthesis. Mutants that fail to continue negative-strand RNA synthesis would be defective in this step, while mutants that continued to make negative strands would be designated as conversion-defective mutants. Cells infected with wt MHV-A59, Alb ts16, and LA ts6 were shifted from 30 °C to 40 °C at 8 hpi; filled bar, 0–20 min pulse; grey bar, 20–40 min pulse; open bar, 40–60 min pulse; dark diagonal bar, 0–30 min pulse; light diagonal bar, 30–60 min pulse.

**Figure 6.** RNA Synthesis Phenotype of the Alb ts16 and LA ts6 Mutants

RNA synthesis (A) or negative-strand RNA synthesis (B) was determined using 20 or 30 min pulse labels with ³H-uridine in the presence of dactinomycin, with or without the addition of CH, after shifting the incubation temperature of MHV-A59-, Alb ts16-, and LA ts6-infected cells from 30 °C to 40 °C at 8 hpi; filled bar, 0–20 min pulse; grey bar, 20–40 min pulse; open bar, 40–60 min pulse; dark diagonal bar, 0–30 min pulse; light diagonal bar, 30–60 min pulse.

Discussion

Taken together with the complementation analysis, the identification of the mutations responsible for the ts phenotypes of Alb ts6, Alb ts16, Alb ts17, Alb ts22, LA ts6, Wü ts18, Wü ts30, and Wü ts38 leads to a number of important conclusions. First, our data strongly suggest that most of the replicase gene products of ORF1a are cis-active and form a single complementation group (cistron I) encompassing, at least, the nsp4 to nsp10 coding region. If correct, our conclusion must mean that a large proportion of nsp1–nsp11 proteins function as a polypeptide, if only initially or transiently, or they associate as a cis-acting complex before they are proteolytically processed. We favor a model in which a pp1a-related polyprotein represents a large modular scaffolding protein that displays binding sites for ORF1b-encoded pp1ab processing products. While the large number of mutants that fall into cistron I clearly suggest that it is extensive and polygenic, it is not yet clear if all of the ORF1a-encoded proteins function in cis. We are aware that the arterivirus Equine arteritis virus ORF1a-encoded protein nsp1 can function in trans [51] and it has recently been shown that the MHV-A59 ORF1a-encoded protein nsp2 is non-essential for virus replication [52]. The genetic analysis of further MHV-A59 ts mutants will be needed to define the precise boundaries of MHV-A59 cistron I.

Second, our results suggest that the replicase gene products encoded in ORF1b (i.e., nsp12–nsp16) are diffusible and thus assemble and function in viral RNA synthesis after cleavage from pp1ab. This also leads us to the prediction that there...
will be five cistrons in ORF1b, each corresponding to one of the proteolytic cleavage products, and we have designated them tentatively as cistrons II–VI in a 5' to 3' direction (nsp12, cistron II; nsp13, cistron III; nsp14, cistron IV; nsp15, cistron V; and nsp16, cistron VI). The idea that the MHV-A59 ORF1b-encoded replicase proteins function in trans is consistent with the results of Brockway et al., who have shown that a green fluorescent protein–tagged MHV-A59 nsp12 is able to diffuse into the replicase-transcriptase complex if expressed individually in virus-infected cells. However, we would also like to note that our data does not exclude the possibility that some of the ORF1b-encoded proteins may function as intermediates, rather than the end products of proteolytic cleavage. For example, functional proteins comprising nsp12/nsp13, nsp13/nsp14, nsp14/nsp15, nsp15/nsp16 as well as nsp13/nsp14/nsp15 could all be accommodated as single cistrons based upon our complementation data. This would lead to the prediction of either three or four cistrons encoded in ORF1b. The idea that a number of the enzymes involved in coronavirus RNA synthesis may be linked not only functionally, i.e., sequentially in a concerted reaction pathway, but also structurally (i.e., as multifunctional proteins) is also suggested by other studies. For example, Ziebuhr and colleagues [53] have shown that 2'-O-ribose-methylated RNA substrates are resistant to cleavage by the SARS-coronavirus endoribonuclease (nsp15), indicating a functional link with the S-adenosylmethionine-dependent 2'-O-methyl transferase (nsp16). We are currently searching for further ts mutants that might help resolve this issue and we are attempting to trans-complement ts mutants with cell lines that constitutively express ORF1b-encoded replicase proteins. Despite these reservations, the genetic data do allow us to conclude that not only nsp5, the 3C-like cysteine protease, and nsp12, the putative RNA-dependent polymerase (as might have been predicted), but also nsp14, the putative MHV exonuclease, nsp16, the putative MHV 2'-O-methyltransferase, nsp4, and nsp10 are essential for the assembly of a functional replicase-transcriptase complex.

In contrast to most other positive-stranded RNA virus, the viral replicase-transcriptase complex of coronaviruses (and most other nidoviruses) functions to amplify the genome via a full-length negative-strand intermediate and to produce, via a discontinuous process, subgenomic-length negative-strand templates that are then copied directly into subgenomic mRNA. How the replicase-transcriptase complex accomplishes these various activities is not understood in any detail. For example, it is not known whether the same replicase-transcriptase complex functions to produce full-length and subgenome-length RNA or how the conversion from negative- to positive-strand RNA synthesis is regulated. Does the analysis of MHV-A59 ts mutants help us to understand these complex processes?

We have shown previously that negative- and positive-strand RNA synthesis occurs simultaneously throughout MHV-A59 infection but that negative-strand synthesis is short-lived, i.e., its synthesis halts within several minutes after protein synthesis is inhibited [24]. This contrasts with positive-strand synthesis, which continues unabated for 1 h and then gradually declines and disappears about 4 h after the inhibition of translation. These observations suggest that unprocessed forms of the replicase polyprotein(s) might function in negative-strand synthesis and that cleavage of the nascent polyprotein inactivates the negative-strand activity of the replicase, as it does for alphaviruses [54,55]. The replicase-transcriptase activity for positive-strand synthesis can be restarted after the block of translation is reversed [27] but, for this to happen, new negative-strand templates need to be synthesized. In other words, it appears that the coronavirus replicase-transcriptase complex ages, losing both its negative-strand templates and its activity. This interpretation fits well with our genetic analysis of the mutants LA ts6, Alb ts16, and Alb ts6, which shows that they all fall into a single complementation group. It is also consistent with our proposal that the replicase proteins encoded in ORF1a are expressed and function as a polyprotein, or that they assemble as a cis-acting complex before they are proteolytically processed. It is also worth noting that in vivo protein labeling experiments indicate that proteolytic processing of both MHV-A59 ORF1a and MHV-A59 ORF1b-encoded replicase proteins is measured in hours rather than minutes [56–58] and that the fully processed 3C-like cysteine protease is first detected several hours post-infection [59], a time at which the rate of viral RNA synthesis is already increasing rapidly [24].

The idea that the MHV replicase-transcriptase complex is active in negative-strand RNA synthesis before pp1a is extensively processed also fits well with our detailed phenotypic analysis of cistron I mutants. In the case of LA ts6, negative-strand synthesis was inhibited after shift to the non-permissive temperature and, in time, this leads to a decline in positive-strand RNA synthesis (unpublished data). Thus, at the non-permissive temperature, LA ts6 could not sustain positive-strand synthesis, nor replace or replenish aging replicase-transcriptase complexes. The causal mutation in LA ts6 would substitute a Glu for the Gln65 residue of wt nsp10. As noted above the Gln65 residue is conserved in Group I, II (including SARScov), and III coronaviruses and its substitution with Glu might prevent the proper folding of pp1a into a conformation that would allow it to participate in the formation of a replicase-transcriptase complex with negative-strand activity. It would be interesting to determine if, at the non-permissive temperature, nsp10 of LA ts6 could associate with nsp12, nsp13, nsp14, nsp15, or nsp16. Also, it was curious that LA ts6 had a very low reversion frequency of \(10^{-8}\). Why certain bases fail to revert at the typical frequency of \(10^{-4}\) to \(10^{-5}\) is unknown but may be indicative of a region of the genome that is transcribed with higher fidelity than other regions. Alternatively, this low reversion frequency may be an inherent property of the LA ts6 replicase-transcriptase complex.

In contrast to LA ts6, Alb ts16 appeared to be able to continue to form negative strands after shift to the non-permissive temperature, but these negative strands were not converted into templates for positive-strand synthesis. We speculate that Alb ts16 has a ts defect in the conversion of the replicase-transcriptase complex from one able to synthesize negative strands to one able to synthesize positive strands. It is certainly suggestive that Alb ts16 had a mutation in nsp5, which is the 3C-like protease of the virus, but it has yet to be determined if this mutation affects the activity of the protease, or if it affects the folding of pp1a or pp1ab, or if the nsp5 C-terminal domain itself could have a function in positive-strand RNA synthesis. Nevertheless, because negative-strand RNA synthesis was inhibited in Alb ts16-infected
cells treated with CH at the time of shift to non-permissive temperature, we propose that the Alb ts16 replicase-transcriptase complex does not retain its activity for minus-strand synthesis. Rather it fails to gain positive-strand synthesis activity at the non-permissive temperature. We favor a model where the activity that makes positive strands is gained at the expense or loss of the activity to make negative strands.

Finally, although we are able to rationalize the genotype of Alb ts22, i.e., a mutation in nsp12 (the RNA dependent RNA polymerase) with its phenotype (i.e., an immediate effect on RNA synthesis at the non-permissive temperature) we were surprised to find that Alb ts17, Wu ts18, Wu ts36, and Wu ts38 also showed the same phenotype but had mutations in other replicase proteins. Generally, it is unusual to find so many ts mutants that show an effect on RNA synthesis if the replicase-transcriptase complex is first allowed to assemble at the permissive temperature. Most RNA-negative ts mutants of alphaviruses, for example, fail to make viral RNA when the infection is initiated at the non-permissive temperature but continue to make viral RNA if shifted to the non-permissive temperature late in infection (unpublished data). One possibility is that nsp14 and nsp16 dissociate or become less tightly associated with the replicase-transcriptase complex after shifting to the non-permissive temperature and this causes the complex to lose elongation activity. Another possibility is that the enzymatic activities associated with nsp14 and nsp16 are altered in the group IV and group VI mutants. Further studies will be required to explain this phenotype.

In summary, our detailed phenotypic analysis of MHV-A59 ts mutants allows us to propose a working model that describes a pathway for viral RNA synthesis in MHV-A59-infected cells. In this model, the replicase-transcriptase complex forms initially and creates a negative-strand template. It is then converted to utilize the negative strand as a template for positive-strand synthesis and, finally, the complex is inactivated by the degradation of negative-strand templates (Figure 7). Our analysis also allows us to place some of our ts mutants at specific points on this pathway. We hope that a more detailed biochemical analysis of these mutants will allow us to identify intermediates in the pathway of RNA synthesis and will provide valuable information of the precise function(s) of the viral replicase proteins involved. Furthermore, we believe that the characterization of these mutants provides an excellent starting point for the generation of second site reversion mutants. This could be done, for example, by using the recently developed MHV reverse genetic system [45] to generate ts mutants with codon, rather than nucleotide substitutions. Second site reversion mutants may then provide valuable information on protein-protein interactions within the replicase-transcriptase complex.

**Materials and Methods**

**Cells and viruses.** Seventeen clone one (17Cl-1) mouse fibroblast cells [60] were cultured at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 6% fetal bovine serum (FBS), 5% tryptose phosphate broth (TPB), penicillin (100 units/ml), and streptomycin (100 μg/ml). Sac(-) cells [61] were cultured at 37 °C in minimal essential medium (MEM) supplemented with 5% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). The A59 strain of MHV and a set of ts mutants derived from MHV-A59 (Alb prefix) were originally obtained from the laboratory of L. Sturman, Wadsworth Center for Laboratories and Research, Albany, New York, United States [40]. Mutants prefixed with LA (Los Angeles) and NC (North Carolina) were obtained from M. Schaaf and R. Baric, University of North Carolina, Chapel Hill, North Carolina, United States and have been initially characterized [39]. Mutants prefixed with Ut (Utrecht) were obtained from W. Spaan, Leiden University Medical Center, Leiden, The Netherlands and have been initially characterized [41]. The LA, NC, and Ut ts mutants were derived from different but related lineages of the Albany isolate of MHV-A59. Mutants prefixed with Wu (Würzburg, Germany) were isolated as described below.

For our studies, virus stocks were derived from the original mutant isolates after plaque purification and propagation in 17Cl-1 cells cultured at 30 °C or 35 °C in low pH DMEM (pH 6.4) containing 6% FBS, 5% TPB, penicillin (100 units/ml), and streptomycin (100 μg/ml) [24]. Revertants were picked from plaques of mutants titered at 39.5 °C, followed by another plaque purification at 39.5 °C. The virus stocks used were first passage and were obtained by using virus from a single plaque (~10^2 pfu) to infect a dish of ~4 × 10^6 17Cl-1 cells to yield 30 ml of stock virus with a titer of 1-6 × 10^3 pfu/ml.

**Isolation of Wu ts mutants.** Sac(-) cells were infected with 10 pfu/cell of MHV-A59 (originally obtained from P. Carthew, Medical Research Council Laboratories, Carshalton, United Kingdom), and incubated for 15 h at 37 °C in medium containing 150 μg/ml of 5-fluorouracil. This concentration of pyrimidine analogue was determined to inhibit virus replication by 80%. The mutagenized virus stock was diluted to 15 pfu/ml in medium and 100 μl aliquots were incubated with 10^5 Sac(-) cells at 30 °C for 48 h. The supernatant was then taken from cultures that displayed syncytium formation and used to infect duplicate cultures of 10^5 Sac(-) cells that were incubated at 30 °C or 39.5 °C for 24 h. The supernatant was then taken from replica cultures that displayed cytopathic effect at 30 °C but not at 39.5 °C, and potential ts mutants were isolated by two plaque purifications. Sequence analysis of the Würzburg strain of MHV-A59 suggests that approximately 8,000 nucleotides at the 5’ end of the genome have been exchanged by recombination with a related but different MHV strain (unpublished data).

**Characterization of mutant stocks for titer and EOP.** 17Cl-1 cells in 60 mm petri dishes were infected with 0.5 ml of 10-fold dilutions of
Two different procedures were used to infect 17Cl-1 cells that had been infected at a MOI of 0.2, producing plaques of equal diameter and Alb 17R cells were mutants. After the above background, while a 4-fold difference above background, a 8 folding complementation, i.e., Alb 17Cl-1 cells were infected with virus, incubated for 13 h at 30 °C to 35 °C in 7.5% CO
. The poly(A)-containing RNA was then isolated from the infected cells using oligo-dT<sub>25</sub> Dynabeads as described previously [42].

**RT-PCR and sequencing.** The entire replica gene-coding region (ORF1a and ORF1b) was sequenced for eight ts mutant and revertant pairs. To do this, we used a set of 121 synthetic oligonucleotides that are complementary to sequences spaced at approximately 350 nucleotide intervals along the positive- and negative-strand copies of the viral RNA (sequences available on request). Five oligonucleotides, P1, P31, P61, P65, and P66, were used to prime the RT of viral RNA with Superscript II RT (Invitrogen, Carlsbad, California, United States). The reaction mix (20 µl), which contained, in addition to pre-supplied buffer, 35 ng of primer, 10–100 ng of viral RNA, 1 mM dNTPs, 10 mM dTT, 25 U of RNAgaurd (Amersham, Little Chalfont, United Kingdom), and 200 U of reverse transcriptase, was incubated at 42 °C for 90 min and then at 94 °C for 2 min. The five cDNA templates were then amplified using eight primer pairs, P1/P6, P2/P22, P3/P30, P4/P38, P5/P45, P6/P53, P7/P60, and P8/P64, and thermostable, recombinant Tag DNA polymerase. The reaction mix (100 µl), which contained in addition to pre-supplied buffer, 70 µg of primer pair, 1 µl of RT reaction product, 200 µM MgCl<sub>2</sub> and 2.5 U of DNA polymerase, was incubated at 94 °C for 1 min, then 94 °C for 20 s, 50 °C for 20 s, 68 °C for 3 min, for a total of 35 cycles and a final 10-min extension at 68 °C. The PCR reaction products were purified by ethanol precipitation using ammonium acetate. Finally, sequence analysis was done using primers P1–P121 and standard cycle sequencing methods. Sequence reaction mixes (10 µl), which contained 70 ng of primer, 100 ng of PCR product, and 3 µl of cycle sequencing mix (BigDye Terminator v3.1, Applied Biosystems, Foster City, California, United States), were incubated at 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min, for a total of 25 cycles. The reaction products were purified by retention on a size exclusion membrane (Montage<sup>™</sup> SEQ100, Millipore, Billerica, Massachusetts, United States) as described by the manufacturer; eluted and analyzed with an ABI 310 Prism Genetic Analyzer. Computer-assisted analysis of sequence data was done using the Lasergene bio-computing software (DNASTAR).

**Supporting Information**

**Figure S1. Plaque Morphology of Alb ts17 Revertants**

The plaque morphologies of Alb <sup>ts17</sup> and Alb <sup>ts17</sup> are illustrated. Alb <sup>ts17</sup> had a reversion (back mutation) frequency of 2 × 10<sup>-3</sup> and there was a mixture of large and small plaques at 40 °C. The virus from the small and large plaques produced progeny that formed uniformly small or large plaques at 40 °C, respectively. At 30 °C, both 17R<sub>1</sub> and 17R<sub>2</sub> produced plaques of equal diameter and Alb 17R<sub>1</sub> produced the same size plaques at 40 °C as the parental or wt MHV- A59. Found at DOI: 10.1371/journal.ppat.0010039.s001 (1.7 MB PPT).

**Table S1. Phenotypic Analysis of MHV-A59 ts Mutants**

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**Author contributions.** S. G. Sawicki, D. L. Sawicki, and S. G. Siddell conceived and designed the experiments, performed the experiments, and analyzed the data. S. G. Sawicki, V. Thiel, and S. G. Siddell wrote the paper.
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