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Evidence for Variable Rates of Recombination in the MHV Genome

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Mouse hepatitis virus has been shown to undergo RNA recombination at high frequency during mixed infection. Temperature-sensitive mutants were isolated using 5-fluorouracil and 5-azacytidine as mutagen. Six RNA^+ mutants that reside within a single complementation group mapping within the S glycoprotein gene of MHV-A59 were isolated which did not cause syncytium at the restrictive temperature. Using standard genetic techniques, a recombination map was established that indicated that these mutants mapped into two distinct domains designated F1 and F2. These genetic domains may correspond to mutations mapping within the S1 and S2 glycoproteins, respectively, and suggest that both the S1 and S2 domains are important in eliciting the fusogenic activity of the S glycoprotein gene. In addition, assuming that most distal ts alleles map roughly 4.0 kb apart, a recombination frequency of 1% per 575–676 bp was predicted through the S glycoprotein gene. Interestingly, this represents a threefold increase in the recombination frequency as compared to rates predicted through the polymerase region. The increase in the recombination rate was probably not due to recombination events resulting in large deletions or insertions (>50 bp), but rather was probably due to a combination of homologous and nonhomologous recombination. A variety of explanations could account for the increased rates of recombination in the S gene.

INTRODUCTION

Mouse hepatitis virus (MHV), a member of Coronaviridae, contains a single-stranded nonsegmented plus-polarity RNA of about 32 kb in length (Lee *et al.*, 1991; Pachuk *et al.*, 1989). The genomic RNA is arranged into seven or eight coding regions and is encapsidated within multiple copies of a 50-kDa nucleocapsid protein (N) (Lai, 1990; Siddell, 1983). The N protein forms a helical nucleocapsid structure that is probably associated with the transcriptional complex (Baric *et al.*, 1988; Compton *et al.*, 1987; Stohlman and Lai, 1979; Stohlman *et al.*, 1988; Sturman *et al.*, 1980). The nucleocapsid structure is surrounded by a lipid envelope and contains two or three virus-specific glycoproteins. The S glycoprotein has a molecular weight of 180 kDa and is frequently cleaved into two 90-kDa glycoproteins designated S1 and S2 (Frana *et al.*, 1985; Sturman *et al.*, 1980, 1985; Sturman and Holmes, 1985). The N-terminal signal sequence is contained within the S1 domain while the C-terminus is contained within the S2 region (Boireau *et al.*, 1990; Luytjes *et al.*, 1987; Rasschaert *et al.*, 1990; Schmidt *et al.*, 1987). The S1 domain can undergo significant amino acid alteration without losing its function while the S2 domain is more highly conserved and contains a hydrophobic heptad repeat element that probably forms a complex coiled coil (Luytjes *et al.*, 1987; Schmidt *et al.*, 1987). The S glycoprotein is responsible for virus binding to the specific cellular receptor, induction of cell fusion, and elicitation of neutralizing antibody and cell-mediated immunity (Collins *et al.*, 1982; Fleming *et al.*, 1986; Sturman *et al.*, 1980, 1985; Sturman and Holmes, 1985). In addition to S, a 23-kDa M glycoprotein is present in the virion that probably functions in assembly and release (Sturman *et al.*, 1980, 1985). In some strains of MHV, a 65-kDa hemagglutinin (HE) that shares considerable sequence homology with the influenza C virus hemagglutinin is also present (Luytjes *et al.*, 1988; Shieh *et al.*, 1989; Yokomori *et al.*, 1989).

Upon entry into susceptible cells, the genomic RNA is translated into one or more polyproteins that act as an RNA dependent RNA polymerase (Mahy *et al.*, 1983; Brayton *et al.*, 1982, 1984). While the exact mechanism for MHV transcription is still under study, the preponderance of data suggest that the genome is initially transcribed into a full-length minus strand RNA, which acts as template for the synthesis of a genome-length RNA and six subgenomic mRNAs by "leader-primed" transcription (Baker *et al.*, 1990; Baric *et al.*, 1983, 1985; Makino *et al.*, 1986a, 1991). In turn, the subgenomic mRNA then act as template for the synthesis of subgenomic minus strands, which participate in successive rounds of mRNA amplification (Sawicki and Sawicki, 1990; Sethna *et al.*, 1989, 1991).

Homologous recombination among viruses with

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nonsegmented RNA genomes has been reported among picornaviruses (Cooper, 1968, 1977; Cooper *et al.*, 1975; King *et al.*, 1982, 1985, 1987; Lake *et al.*, 1975), brome mosaic virus (Bujarski and Kaesberg, 1986), alphaviruses (Hahn *et al.*, 1988; Weiss and Schlesinger, 1991), cowpea chlorotic mottle virus 3a (Allison *et al.*, 1990), and coronaviruses (Keck *et al.*, 1988a,b; Lai *et al.*, 1985; Makino *et al.*, 1986b). During MHV replication, the RNA recombination frequency is unusually high and approaches 25% or more during mixed infection (Baric *et al.*, 1990). Recently, biochemical analysis has revealed extensive polymorphisms and deletions in the sequence of the S glycoprotein gene of different MHV variants. The variant viruses were probably derived from nonhomologous recombination because no consensus or conserved sequences flanked the deletions (Banner *et al.*, 1990; Parker *et al.*, 1990). Sequence analysis of RNA recombinant viruses also suggests the presence of a recombination "hot spot" in the MHV S glycoprotein gene (Banner *et al.*, 1990). However, in the absence of selection pressure, RNA recombination sites in this region are random, suggesting that this preferred site reflects the selection for biologically more efficient recombinants (Banner *et al.*, 1991).

Recombination analysis provides a powerful tool for mapping genetic loci and determining the recombination frequency between individual temperature sensitive (ts) mutations (Baric *et al.*, 1990; Cooper, 1968, 1977; Lake *et al.*, 1975). In this study, several group F RNA^+ ts mutants were used to establish a genetic recombination map in the S glycoprotein gene of MHV-A59.

MATERIAL AND METHODS

Virus propagation and assay procedures

Temperature-sensitive mutants representing the group F RNA^+ mutants (LA7, LA12, NC6, NC14, NC16, NC17) and group E RNA^- mutants (LA18) of MHV-A59 were used throughout the course of this study. All virus stocks were propagated at 32° in 150-cm^2 flasks containing DBT cells as described previously (Schaad *et al.*, 1990). Plaque assays were performed at either the permissive (32°) or nonpermissive (39.5°) temperatures in DBT cells in Dulbecco's modified essential medium (DMEM) (Sigma) containing 10% Nu-serum (Collaborative Research, Inc.), 1% gentamycin/kanamycin (GIBCO), and 0.8% agarose (BRL). All plaque assays were stained 28–36 hr postinfection by the addition of neutral red for 2 hr. Cloned 17Cl-1 cells were kindly provided by Dr. Sue Baker (Loyola University, Chicago) and maintained at 37° in DMEM containing 7% newborn calf serum (GIBCO), 1% gentamycin/kanamycin, 2% glucose (Sigma), and 5% tryptose-phosphate broth (Sigma).

Mutagenesis and isolation of revertant and recombinant virus stocks

Most of the mutants used in this study have been characterized previously (LA18, LA7, LA12, NC6) (Schaad *et al.*, 1990). To increase the accuracy of our recombination mapping data, additional mutants residing within complementation group F were isolated. Briefly, cultures of MHV-A59-infected cells were mutagenized with either 20 μg/ml 5-azacytidine or 100 μg/ml 5-fluorouracil for 12 hr. Supernatant fluids containing mutagenized virus were plaqued at permissive temperature and individual plaques were isolated. Each plaque was diluted into 0.5 ml ice-cold PBS, screened at 32° and 39.5° by plaque assay, and isolates displaying ts phenotypes were repurified two to three times at 32°. Virus stocks were assayed at the permissive and restrictive temperatures and only those isolates displaying reversion frequencies of less than 10^{-3} were used for future studies. Complementation and RNA phenotype analysis were performed as described previously (Leibowitz *et al.*, 1982; Martin *et al.*, 1988; Schaad *et al.*, 1990), and three additional group F RNA^+ mutants were isolated and designated NC14, NC16, and NC17. NC14 and NC16 were isolated using 5-fluorouracil, while NC17 was isolated using 5-azacytidine.

To select for revertants from the group F RNA^+ ts mutants, virus stocks were plaque assayed at 39.5°, and individual plaques were isolated and repurified by plague assay at the restrictive temperature. Individual stocks of revertant virus were grown in 150-cm^2 flasks at 37° and the reversion frequency of each was determined by plague assay at 32 and 39.5°. At least three independent revertants from each RNA^+ mutant were isolated. To isolate RNA recombinant virus, DBT cells were coinfected with two different ts mutants at an m.o.i. of 10 each at 32°. The medium was harvested at 16 hr postinfection and stored at −70°. Recombinant viruses were isolated at the restrictive temperature, repurified at 39.5°, and grown in DBT cells at 37°.

Radiolabeling of viral mRNAs and temperature shift experiments

Culture of 17CL-1 cells (1×10^6) in 25-mm six-well dishes (Costar) were infected with various group F ts mutants, revertants, or recombinant viruses at a multiplicity of infection of 10 for 1 hr at room temperature. Following adsorption, the inoculum was removed, and the cultures were rinsed with PBS and incubated in complete DMEM for 4 hr at 32°. Actinomycin D (2 μg/

ml) was added for 1 hr and one-half the cultures shifted to the restrictive temperature at 5 hr postinfection by the addition of prewarmed media. Viral progeny were harvested at varying intervals and analyzed by plaque assay.

To determine whether these mutants could transcribe viral mRNA, cultures of cells were infected with various ts mutants and maintained at the permissive or restrictive temperature for 8 hr. The media were removed and the cultures washed with 2 ml of ISO–TKM (10 m*M* Tris–HCl, pH 7.5, 150 m*M* KCl, and 1.5 m*M* $MgCl_2$). The RNA was isolated as described by Sawicki and Sawicki, 1990, and extensively purified by successive rounds of phenol (U.S. Biochemical Company), phenol/chloroform/isoamy alcohol, and chloroform extraction. The RNA was bound to nitrocellulose filters and probed with radiolabeled RNA probes specific for the MHV N gene (Schaad *et al.,* 1990). The blots were washed and exposed to XAR-5 film for 24 hr.

Recombination analysis and establishing a genetic recombination map of the group F RNA$^{\pm}$ mutants

Recombination analyses were performed as previously described (Baric *et al.,* 1990) and the recombination frequencies calculated as the percentage of ts$^+$ virus present in the progeny from the following formula:

$$RF = \frac{(AB)_{39.5} - (A + B)_{39.5}}{(AB)_{32}} \times 100\%.$$

$(AB)_{39.5}$ was the titer of the cross at nonpermissive temperature while $(AB)_{32}$ represented the titer of the same cross at permissive temperature. $(A + B)_{39.5}$ was the sum of the revertants of each parent strain assayed at the nonpermissive temperature. The formula only measured single or odd-number cross-over events resulting in the ts$^+$ phenotype and did not account for recombination events that resulted in the double-ts mutant phenotype. All recombination frequencies were standardized to a standard cross [LA7 × NC16; recombination frequency 3.0 ± 1.2] as previously described (Baric *et al.,* 1990). The mutants were aligned according to their standardized recombination frequencies and positioned from LA7, LA18, and NC16.

PCR amplification of the MHV S gene

Cultures of DBT cells in 100-mm^2 dishes were infected at a m.o.i. of 5 with different ts mutants, revertant viruses, or recombinant viruses, and intercellular RNA was harvested at 8–12 hr postinfection. The precipitate was resuspended in TE buffer (10 m*M* Tris–HCl, pH 7.2, 1 m*M* EDTA) and used as template for reverse transcription and TAQ amplification reactions.

cDNA synthesis was carried out in a 20-μl reaction mixture containing 1 μg intercellular RNA, 5 m*M* $MgCl_2$, 10 m*M* Tris–HCl pH 8.4, 50 m*M* KCl, 0.1% Triton X-100 (Sigma), 1 m*M* dNTP each (Promega), 0.5 μg random hexamer (Perkin Elmer Cetus), 1U/μl RNasin (Promega), 15 U AMV reverse transcriptase (Promega) and incubated at 42° for 1 hr. The products were extracted and precipitated in ethanol and resuspended in 50 μl H_2O prior to successive rounds of polymerase chain amplification (PCR).

One-tenth of the cDNA was amplified in a 50 μl reaction mixture containing 10 m*M* Tris–HCl (pH 9.0), 50 m*M* KCl, 3 m*M* $MgCl_2$, 0.1% Triton X-100, 1.25 U TAQ DNA polymerase (Promega), 0.2 m*M* dNTP mixture and the appropriate primer pairs (100 ng of each). PCR amplification was performed for 30 successive cycles at 1.5 min at 94° to denature the DNA, 2 min at 55° for primer annealing, and 4 min at 72° for primer extension. The products were loaded onto 1% agarose gels in TAE (40 m*M* Tris, 9 m*M* NaCl, 1 m*M* EDTA, (pH 8.0)), separated electrophoretically, stained with ethidium bromide, and visualized under UV light. The DNA products were transferred to nitrocellulose filters, and confirmed by hybridization with ^{32}P-labeled oligomer probes specific for internal sequences in each gene fragment amplified.

Three sets of overlapping primer pairs were obtained from highly conserved sequences in the MHV-A59 and JHM S glycoprotein genes (Banner *et al.,* 1990; Luytjes *et al.,* 1987; Parker *et al.,* 1990). Set A was derived from sequences spanning nucleotides −1 to 15 (CAT GCT GGT CGT GTT T) and 662–677 (AAC GTA GTA GCG GAG G), respectively, and should result in a DNA fragment of 0.67 kb in length. Set B was derived from nucleotides 642–662 (GCG TAC TAT TCG GAT AAA CC) and 2103–2115 (CCC ACG ACC GAA TAC G) and were used to amplify a ~1.5-kb fragment encompassing the hypervariable region in the S1 domain (Banner *et al.,* 1990; Parker *et al.,* 1990). Set C was derived from nucleotides 2067–2083 (ACG GAT GAG GCG CTT C) and 4055–4070 (GTC TTT CCA GGA GAG G) and were used to amplify a ~2.0-kb fragment spanning the C terminus of the S glycoprotein gene. Internal oligodeoxynucleotides were used as probe to demonstrate the specificity of the PCR reaction.

RESULTS

Isolation and characterization of the group F RNA$^+$ Ts mutants and revertants

Six complementation group F RNA$^+$ mutants and one group E RNA$^-$ mutant were used during the course of this study. Three of the RNA$^+$ mutants (LA7, LA12,

TABLE 1

PHENOTYPIC CHARACTERIZATION OF RNA$^+$ TEMPERATURE-SENSITIVE MUTANTS AND THEIR REVERTANTS

Designation	Titer (PFU/ml) 32°	Titer (PFU/ml) 39°	Reversion frequency[a]	RNA phenotype
Mutant				
LA7	3.0×10^7	1.0×10^3	3.3×10^{-4}	+
LA12	1.1×10^8	1.0×10^2	1.0×10^{-6}	+
NC6	5.5×10^7	1.3×10^2	2.4×10^{-6}	+
NC14	5.5×10^7	9.5×10^4	1.7×10^{-3}	+
NC16	3.5×10^7	4.3×10^3	1.2×10^{-4}	+
NC17	7.8×10^7	1.4×10^4	1.8×10^{-4}	+
Revertant				
LA7-R1	6.9×10^7	8.7×10^7	1.2×10^0	+
LA7-R2	7.3×10^7	7.2×10^7	1.0×10^0	+
LA7-R3	9.1×10^7	9.5×10^7	1.0×10^0	+
LA12-R1	2.1×10^7	2.0×10^7	9.5×10^{-1}	+
LA12-R2	8.5×10^6	5.5×10^6	6.4×10^{-1}	+
LA12-R3	9.1×10^7	7.2×10^6	7.9×10^{-2}	+
NC6-R1	2.2×10^7	2.4×10^7	1.1×10^0	+
NC6-R2	4.0×10^7	2.0×10^6	5.0×10^{-2}	+
NC6-R3	8.6×10^7	8.4×10^7	9.7×10^{-1}	+
NC14-R1	1.2×10^8	1.2×10^8	1.0×10^0	+
NC14-R2	1.2×10^8	1.2×10^8	1.0×10^0	+
NC14-R3	9.4×10^7	1.2×10^8	1.2×10^0	+
NC16-R1	8.2×10^7	9.2×10^7	1.1×10^0	+
NC16-R2	1.0×10^8	9.0×10^7	9.0×10^{-1}	+
NC16-R3	9.2×10^7	8.5×10^7	9.2×10^{-1}	+
NC17-R1	2.8×10^7	3.2×10^7	1.1×10^0	+
NC17-R2	5.2×10^7	4.5×10^7	8.6×10^{-1}	+
NC17-R3	4.7×10^7	4.6×10^7	9.7×10^{-1}	+
NC17-R4	5.9×10^7	4.8×10^7	8.1×10^{-1}	+

[a] Calculated as 39.5/32°.

NC6) and one RNA$^-$ mutant (LA18) have been characterized previously (Schaad *et al.*, 1990). To increase the accuracy of the recombination map and obtain more representative mutants in the S glycoprotein gene, three additional RNA$^+$ mutants were isolated by mutagenesis as previously described (Schaad *et al.*, 1990). Two mutants were isolated following mutagenesis with 100 μg/ml 5-fluorouracil (NC14, NC16) and one mutant with 20 μg/ml 5-azacytidine (NC17). All mutants had reversion frequencies of less than 10^{-3}, and did not complement each others defect at the restrictive temperature (Table 1, data not shown). All of the RNA$^+$ mutants were capable of transcribing viral mRNA when maintained at the restrictive temperature (data not shown, Table 1). While NC14 produced a small amount syncytium, the remainder of the mutants were not capable of giant cell formation at the restrictive temperature (Fig. 1).

To assist in precisely defining the location of these mutations and examining the mechanism for MHV RNA recombination within the S glycoprotein gene, a panel of revertants were obtained from six of the RNA$^+$ mutants (Table 1). Revertant viruses had similar titers, produced syncytium, and were of the RNA$^+$ phenotype when assayed at both permissive and restrictive temperatures. To conclusively document the revertant phenotype and determine if growth characteristics were similar to parental controls, growth curves were compared at the permissive and restrictive temperatures. Cultures of cells were infected with NC16 or its revertant, NC16-R1, at 32° and shifted to 39.5° at 5.0 hr postinfection. Viral progeny were harvested at different times after infection and assayed by plaque assay at the permissive temperature. Similar growth curves were evident between ts NC16 and its revertant NC16-R1 in cultures maintained at the permissive temperature (Fig. 2). However, following temperature shift, growth of ts NC16 was inhibited significantly under conditions in which the revertant replicated normally. Similar results have also been obtained with other mutants and their revertants (data not shown).

Recombination versus reversion frequencies between the group F RNA$^+$ mutants

To assess the ability of these mutants to be used in recombination studies, we performed a series of crosses between different group F ts mutants and examined the differences between the recombination and reversion frequencies. Cultures of cells were infected at a m.o.i. of 10 each with two different ts mutants and maintained at 32° for 14 hr postinfection. The progeny were harvested and titered at the permissive and restrictive temperatures. Recombination frequencies ranged from as little as 0.1% to >4.0% suggesting that the mutants map in different locations in the gene. The ratio of recombinants to revertants ranged from 9 to 3140 times higher than the sum of the spontaneous reversion frequencies of each individual ts mutant used in the cross and indicated that recombination mapping was feasible throughout this region (Table 2). The extent of the difference in the ratio of recombinants to revertants probably reflected the stability of the mutants used in the cross and the distance between the individual ts lesions.

Establishing a recombination map for the group F RNA$^+$ mutants

In contrast to poliovirus and apthovirus ts mutants, MHV-A59 ts mutants were amenable to complementa-

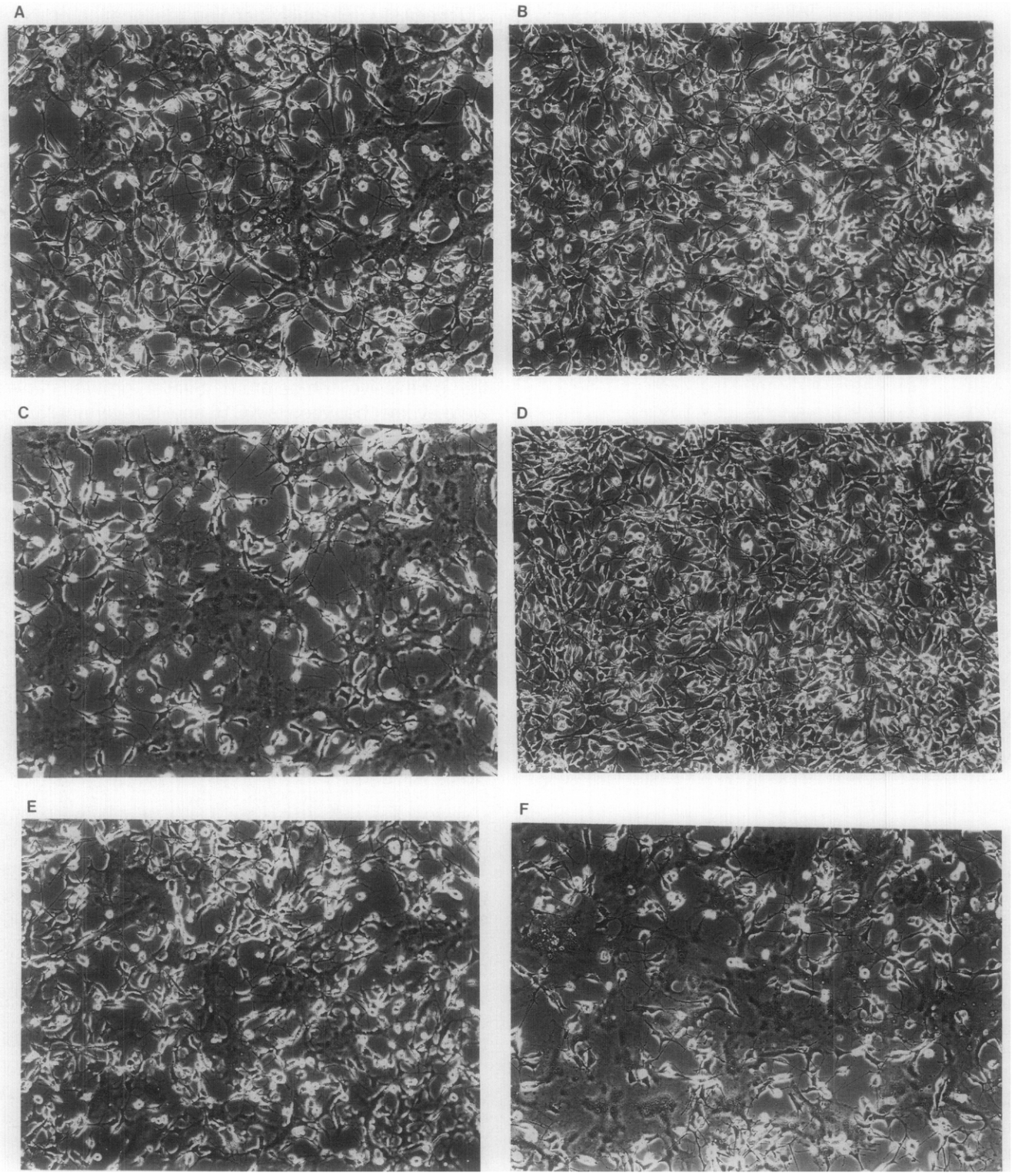

FIG. 1. Syncytium formation at the permissive and restrictive temperatures. Cultures of DBT cells were infected at a m.o.i. of 5 for 1 hr with NC6, NC16, or various revertants. The viral inoculum was removed and the cultures overlaid with media prewarmed at the permissive (32°) or restrictive temperatures (39.5°). Infected cultures were maintained at the permissive or restrictive temperatures for 11 hr and photographed. (A) NC6-32°; (B) NC6-39.5°; (C) NC16-32°; (D) NC16-39.5°; (E) NC6R1-32°; (F) NC6R1-39.5°.

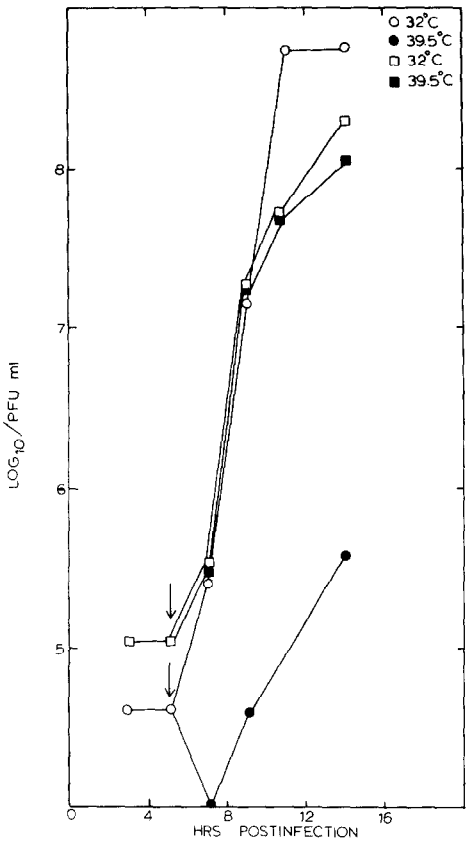

FIG. 2. Virus growth curves following shift to restrictive temperature. Duplicate cultures of cells were infected with group F mutants NC16 or its revertant NC16-R2 at a m.o.i. of 10 and incubated at permissive temperature for 5 hr. One-half the cultures were shifted to restrictive temperature by the addition of prewarmed medium and virus progeny were harvested at the indicated times. (○) NC16-32°; (●) NC16-39.5°; (□) NC16R1-32°; (■) NC16R1-39.5°.

tion analysis, providing strong evidence that all of the RNA$^+$ ts mutants used in this study contain defects in a single gene or a noncomplementable function (Koolen *et al.*, 1983; Leibowitz *et al.*, 1982; Martin *et al.*, 1988; Schaad *et al.*, 1990).

All mutants were originally crossed three to five times with the reference mutants LA7, NC16, or a group E RNA$^-$ mutant LA18 and standardized to the reference cross LA7 × NC16. To obtain a more detailed map of the group F mutants, crosses were also performed among each of the viruses used in this study and standardized to the reference cross. The results of these experiments are shown in Table 3. The distances between different ts mutants were within statistical limits and permitted the construction of a genetic map (Fig. 3). On the basis of their genetic recombination frequencies, the group F RNA$^+$ ts mutants appeared to map into two distinct domains, designated F1 (LA7, LA12, NC6) and F2 (NC14, NC16, NC17, LA12). Ts LA12 mapped closely to representative mutants within each subgroup suggesting that it may be a double mutant.

Assuming that crossover events occur in both directions and that NC6/LA7 and NC14/NC16 map at different ends of the 4.0-kb S glycoprotein gene, a 1% recombination frequency occurred over 575–667 nucleotide pairs of RNA. Since these mutants do not produce syncytium at the restrictive temperature (Fig. 1) and sequence analysis of RNA recombinant viruses suggested that the defect in LA7 mapped within the first 1.1 kb of the S glycoprotein gene (Banner *et al.*, 1990; Keck *et al.*, 1987, 1988a,b; Makino *et al.*, 1986b, 1987), these data provided an anchor for mapping the location of the remaining group F RNA$^+$ mutants. These data predicted that the F1 mutants map in the S1 glycoprotein while the F2 mutants map within the S2 glycoprotein sequences (Fig. 4).

Comparisons between the recombination frequencies in the polymerase and S glycoprotein genes

Using well characterized ts mutants of MHV-A59, we have predicted that the recombination frequency between the group F RNA$^+$ mutant LA7, and the group A RNA$^-$ mutants LA6/LA3 to be approximately 1%/1300–1400 nucleotides of dsRNA (Baric *et al.*, 1990) (Fig. 5). To determine if recombination frequencies vary in different portions of the genome, we compared the maximum recombination frequencies between mutants spanning the polymerase genes of MHV (Group A (LA3/LA6) × Group E (LA18)) across gene B encoding the p30 and HE genes (Group E (LA18) × Group F (LA7/LA12)) or through the S glycoprotein gene (Group F (LA7 × NC16/NC14)). All mutants were crossed three to five times and average recombination frequencies calculated as previously described (Baric *et al.*, 1990) (Fig. 5). Interestingly, the maximum recombination frequency predicted between the two most distant ts mutants in complementation groups A (LA3, LA6) and E (LA18) in the 22-kb polymerase region ranged from about 1%/1800 (LA18 × LA3) to 1%/2500 (LA18 × LA6) nucleotides of ds RNA. The maximum recombination frequency predicted between the group E and F mutants that spanned the p30 and HE genes ranged from about 1%/1100 (LA12 × LA18) to 1%/1400 (LA7 × LA18) nucleotides of dsRNA. Since the maximum predicted recombination frequency across the 4.0-kb S glycoprotein gene ranged from about 1%/575 (LA7 × NC14) to 1%/667 (LA7 × NC16) nucleotides of dsRNA, these data suggested that variable rates of recombination occur within different portions of the MHV genome (Figure 5).

TABLE 2

RECOMBINATION VS REVERSION FREQUENCY AMONG THE GROUP F RNA$^+$ MUTANTS

Mutant	(PFU/ml) 32°	39.5°	Reversion frequency[a]	Recombination[b] Frequency (%)	Prop[c]
LA7	3.3×10^7	1.0×10^2	1.3×10^{-5}	—	—
LA12	1.9×10^7	2.5×10^2	1.8×10^{-4}	—	—
NC6	1.9×10^7	3.5×10^3	1.8×10^{-4}	—	—
NC14	5.0×10^7	5.3×10^3	1.0×10^{-4}	—	—
NC16	8.5×10^7	2.3×10^4	2.7×10^{-4}	—	—
NC17	2.5×10^7	3.2×10^4	1.2×10^{-3}	—	—
Cross					
LA7 × LA12	6.5×10^7	1.8×10^5	—	0.27	514
LA7 × NC6	4.9×10^7	3.0×10^4	—	0.06	9
LA7 × NC14	1.5×10^8	5.0×10^6	—	3.32	500
LA7 × NC16	1.6×10^8	5.4×10^6	—	3.30	200
LA7 × NC17	1.5×10^8	3.8×10^6	—	2.60	3140
NC16 × NC6	1.5×10^8	5.9×10^6	—	4.07	227
NC16 × NC17	1.2×10^8	5.4×10^5	—	0.43	10

[a] Calculated as 39.5/32°.
[b] Recombination frequency was calculated as described under Methods.
[c] Proportion is the number of ts$^+$ recombinant viruses divided by the sum of the revertant viruses titered at 39.5° from singly infected cells.

Are deletions contributing to the increased rates of recombination among the group F RNA$^+$ mutants?

Extensive amounts of polymorphism and deletion have been detected in both the HE and the S glycoprotein gene sequences of different MHV strains and in the sequence of other coronaviruses (Banner *et al.*, 1990; Boireau *et al.*, 1990; La Monica *et al.*, 1991; Parker *et al.*, 1990; Rasschaert *et al.*, 1990). It is possible that the increased rate of recombination within the S glycoprotein gene may represent the sum of homologous recombination and recombination (homologous or nonhomologous) resulting in deletions. To test this possibility, cultures of cells were infected with various combinations of ts mutants as shown in Fig. 4 and RNA recombinant viruses isolated 14 hr postinfection. Since the ratio of recombinants/revertants ranged from 9 to 3140 in these crosses (Table 2), these data suggested that the majority of ts$^+$ isolates represented true RNA recombinants and not revertants. Based on the recombination rate in the S glycoprotein gene, the predicted distance between the ts alleles used in these crosses ranged from ~100 bp to >1000 nucleotides (Fig. 4).

To determine if deletions were present within the S gene of RNA recombinant viruses, a series of overlapping primers were synthesized that span the entire S glycoprotein gene of MHV-A59. The location of these primers as well as the size of predicted PCR products are shown in Fig. 4. Cultures of cells were infected with various RNA recombinant viruses and intercellular RNA was harvested at 8–12 hr postinfection. Following cDNA synthesis and 30 rounds of amplification with the TAQ polymerase and specific oligonucleotide primer pairs, the DNA products were separated on 1%

TABLE 3

GENETIC RECOMBINATION FREQUENCIES[a] AMONG THE GROUP F RNA$^+$ MUTANTS

Mutant	LA7	LA12	NC6	NC14	NC16	NC17	LA18
LA7	—	0.2 ± .02	.2 ± .01	3.4 ± .5	3.0 ± 1.2	1.7 ± .7	1.2 ± .3
LA12	—	—	.4 ± .03	ND[b]	<0.1	<0.1	1.6 ± .4
NC6	—	—	—	2.4 ± .5	2.8 ± .9	2.3 ± .7	0.9 ± .2
NC14	—	—	—	—	.2 ± .09	.4 ± .2	5.5 ± 3
NC16	—	—	—	—	—	0.3 ± .1	4.6 ± .6
NC17	—	—	—	—	—	—	3.5 ± .8

[a] Genetic recombination frequencies were calculated as described in the text (N = 3 to 5).
[b] ND, Not done.

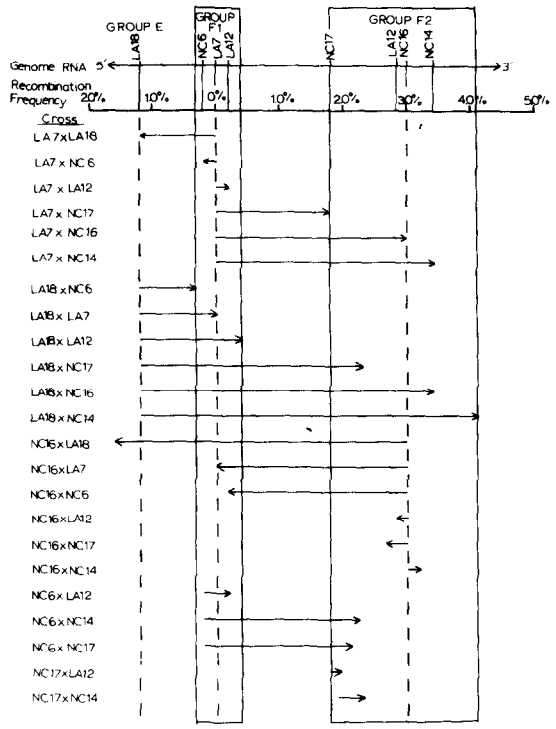

FIG. 3. Establishing a genetic recombination map for MHV-A59 complementation group F. Cultures of DBT cells were infected with different combinations of MHV-A59 ts mutants at a m.o.i. of 10 each and the recombination frequency determined as described under Material and Methods. All mutants were originally crossed and positioned with respect to LA7, LA18, and NC16. Percent recombination frequency is shown across the top of the figure and the solid arrows represent the calculated recombination frequency between each cross. The boxed areas represent the predicted domains of the Group F subgroups F1 and F2. LA12 appears to represent a double mutant since it maps in both the F1 and F2 domains.

agarose gels and visualized by UV light or southern blotting techniques (Fig. 6). In Fig. 6a, PCR products spanning the hypervariable region of MHV-A59, JHM, and MHV2 clearly demonstrate the 89 amino acid deletion that is present in the JHM S glycoprotein gene (Banner *et al.*, 1990; Parker *et al.*, 1990). Southern blotting techniques using an internal oligomer probe demonstrate that the ~1.5-kb fragment is specific for the MHV S glycoprotein sequences spanning nucleotides 642–2115 and is not present in uninfected cells (Fig. 6b). No obvious deletions were detected through the hypervariable region (Fig. 6c), N-terminus, or C terminus in the S glycoprotein gene of over 20 different RNA recombinant viruses tested (Fig. 6d). Since the distance between the group F1 and F2 mutants would have required sizable deletions (>100 bp) to result in ts^+ virus (Fig. 4), these data suggested that large deletions were rare events in these crosses, and did not contribute to an increase in the recombination frequency within the S glycoprotein gene.

These studies cannot rule out the possibility of small deletions in one ts parent contributing to an increase in the ts^+ progeny. However, no obvious deletions were detected in the S glycoprotein gene of 19 different revertant viruses. In Fig. 6e, PCR products spanning the hypervariable region of NC17 and its four revertants are presented. While these data cannot conclusively rule out the possibility of small deletions (<50 bp) that would not be detected under these conditions, the data suggested that most revertants probably contain a single nucleotide reversion.

DISCUSSION

Although homologous recombination occurs during the replication strategy of several nonsegmented RNA viruses (Bujarski and Kaesberg, 1986; Cooper, 1968; Hahn *et al.*, 1988; King *et al.*, 1987; Weiss and Schlesinger, 1991), high frequency RNA recombination is probably a unique phenomenon associated with MHV and perhaps the replication strategies of other coronavirus (Baric *et al.*, 1990; Keck *et al.*, 1987, 1988a,b; Makino *et al.*, 1986b, 1987; Lai *et al.*, 1985). Using highly characterized ts mutants of MHV-A59, we established a genetic recombination map in the polymer-

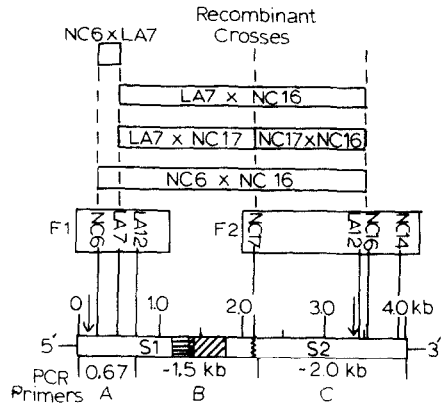

FIG. 4. Predicted nucleotide domains of the group F $RNA^{\pm}$ mutants. Assuming a recombination frequency of 1%/575 bp of dsRNA and that LA7 maps roughly 0.5 kb from the 5' end of the S glycoprotein gene, the tentative nucleotide domains of the mutants mapping in the F1 and F2 subgroups were predicted. The hatched boxes represent the location of the region of polymorphism and putative hot spot of recombination in the MHV genome (Banner *et al.*, 1990; Parker *et al.*, 1989). Arrows represent the approximate location of neutralizing epitopes in the S glycoprotein (Routledge *et al.*, 1991; Weissmiller *et al.*, 1990). To determine if recombination events resulting in deletions were contributing to the increased recombination rate in the S glycoprotein gene, a series of crosses were obtained between different RNA^+ mutants as shown at the top of the figure. PCR products spanning different portions of the MHV S glycoprotein gene were obtained using specific oligomer products and the TAQ polymerase. The location of these primer pairs and their predicted products are shown in the bottom of the figure.

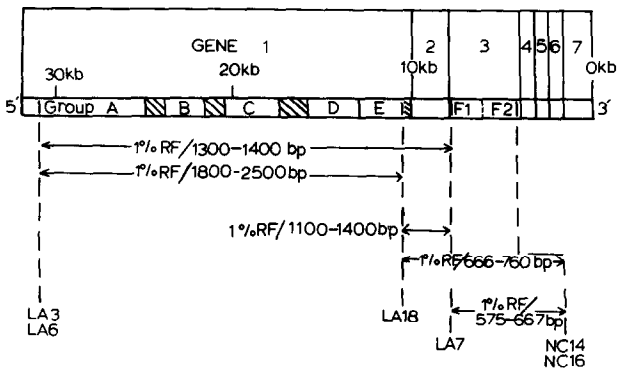

FIG. 5. Comparison of the recombination frequencies through different regions of the MHV-A59 genome. Cultures of cells were infected with different ts mutants at a m.o.i. of 10 each and maintained at the permissive temperature for 14 hr. Recombination rates were compared by calculating the recombination frequencies between mutants spanning different portions of the MHV genome. To predict the maximum recombination frequency at the 5′ end of the genome, we calculated the recombination frequency between the three most distant RNA$^-$ mutants (Group A RNA$^-$ mutants: LA3, LA6; Group F RNA$^-$ mutant: LA18) assuming that these mutants map at the most distant ends of the 22-kb polymerase region (~21 kb apart). To map the maximum recombination rate across the HE and p30 genes, recombination rates were determined between mutants mapping approximately 3.5 kb apart at the 3′ end of the polymerase gene (LA18) and the 5′ end of the S glycoprotein gene (Group F RNA$^+$ mutants: LA7, LA12). Recombination rates were calculated through the S glycoprotein gene, assuming the group F RNA$^+$ mutants LA7 and NC14/NC16 map roughly 4.0 kb apart.

ase genes of MHV. Assuming that the recombination frequency was equivalent throughout the entire genome, these data suggested that the recombination frequency approached 25% or more (Baric *et al.*, 1990). In this study, we have demonstrated that the ts mutants from a RNA$^+$ complementation group of MHV-A59 can also be arranged into an additive, linear, genetic map.

Several lines of evidence strongly suggest that the group F RNA$^+$ mutants map in the S glycoprotein gene of MHV-A59. First, the group F mutants do not produce syncythium when maintained at the restrictive temperature. Induction of cell fusion has been mapped to either the S2 domain of the bovine coronavirus S glycoprotein gene (Yoo *et al.*, 1991) or the MHV S1 and S2 glycoprotein domains (Gallagher *et al.*, 1991; Routledge *et al.*, 1991; Weismiller *et al.*, 1990). Second, T1 fingerprint analysis of RNA recombinant viruses derived from crosses between ts LA7 or LA12 and MHV-JHM demonstrated that the MHV-A59 S1 domain was always replaced by heterologous MHV-JHM sequences. Since no other region in the MHV-A59 genome was uniformly replaced in recombinant viruses, these data strongly suggested that the mutations in these ts viruses reside within the S1 coding sequences (Keck *et al.*, 1987, 1988a,b; Makino *et al.*, 1986b, 1987). Finally, sequence analysis of RNA recombinant viruses derived from these mutants place the ts allele in LA7 within the 5′ most 1.1 kb of the S1 glycoprotein gene (Banner *et al.*, 1990). While these data do not definitively prove that the Group F mutants map in the S glycoprotein gene sequence, these data have strongly supported the localization of these mutations within this coding region.

On the basis of recombination mapping data, the group F RNA$^+$ mutants appear to map into two discrete domains in the S glycoprotein gene sequence (Fig. 4). Data from our laboratory and others suggest that the F1 mutants probably map within the S1 glycoprotein while the F2 mutants map within the S2 glycoprotein sequences (Banner *et al.*, 1990; Keck *et al.*, 1987, 1988a,b; Makino *et al.*, 1987, 1988). Sequence analysis of the group F RNA$^+$ mutants and revertant viruses will be required to definitively map the location of these alleles. However, since mutants from both subgroups are incapable of producing syncytium at the restrictive temperature, these data suggest that both domains are important in eliciting cell fusion. Currently, it is unclear whether the alterations in the F1 and F2 conditional lethal mutants result in a temperature-sensitive fusogenic domain or alter the synthesis, transport, and surface expression of the S glycoprotein gene.

The recombination frequency for the entire MHV genome has been estimated to approach 25% or more (Baric *et al.*, 1990). These estimates were based on the assumption that the recombination frequency was uniform and approached 1%/1300–1400 nucleotides of dsRNA throughout the entire 32-kb MHV genome. However, recombination rates measured within the polymerase region and S glycoprotein gene were estimated to occur at frequencies of about 1%/1800–2500 and 1%/575–667 bp of dsRNA, respectively (Fig. 5). Recombination rates across the p30/HE genes were estimated to occur at about 1%/1100–1400 bp of dsRNA. Currently, our best estimate for an MHV recombination frequency was measured within the S glycoprotein gene because these mutations must have resided within a 4.0-kb stretch of RNA. Moreover, the frequency of recombination was internally consistent between several independent crosses within a single complementation group (Table 3). While definitive proof will require the identification of the exact location of the ts alleles used in these studies, these data suggest that the recombination frequency varies in different portions of the genome and is roughly threefold higher in the S glycoprotein gene.

Several explanations could account for an increase in the recombination frequency within the S glycoprotein gene. First, the recombination rate through the

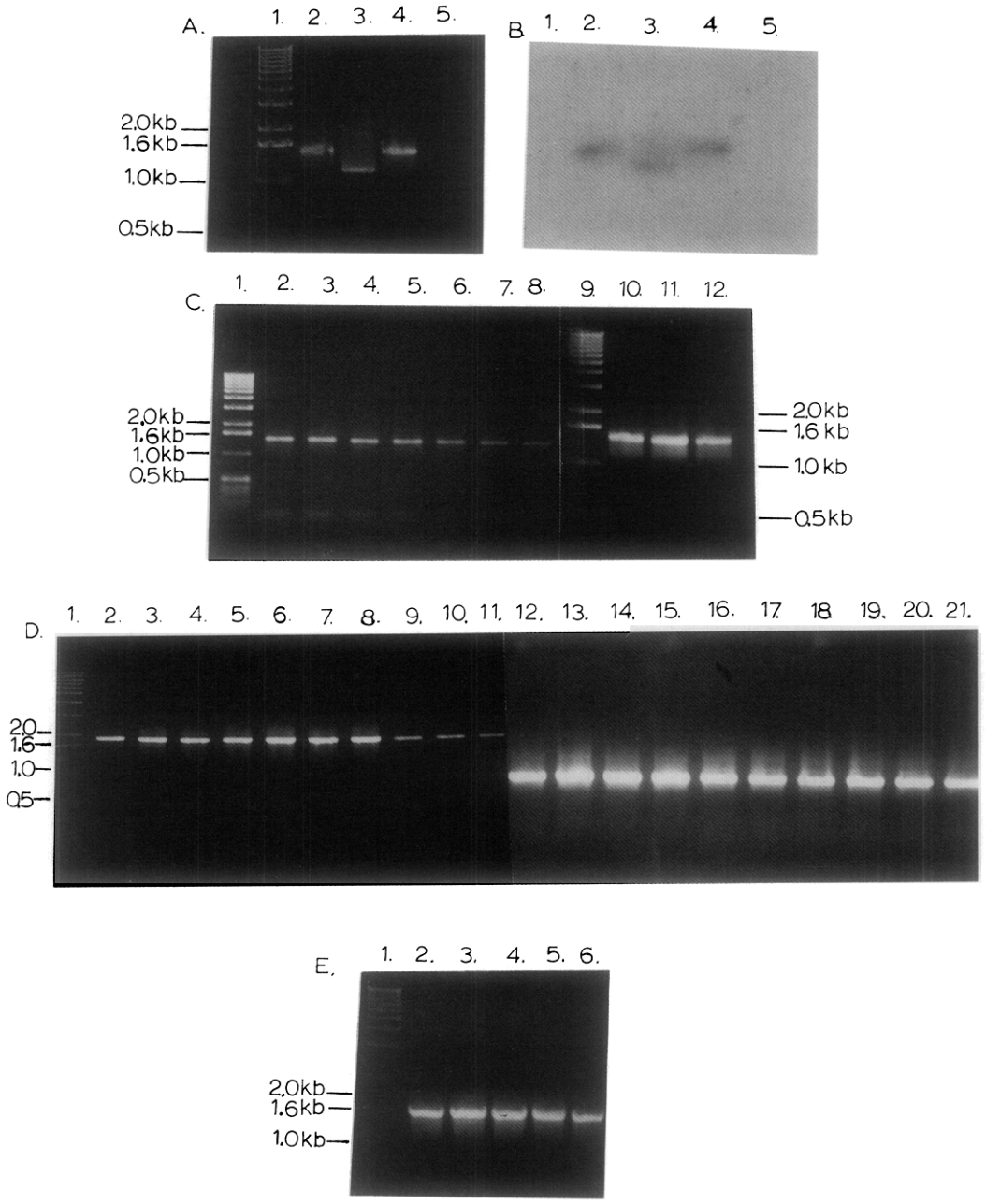

FIG. 6. PCR amplification and size analysis of the S glycoprotein gene of wildtype, Ts, recombinant, and revertant viruses. Cultures of cells were infected with different strains of MHV ts mutants, recombinant viruses, or revertant viruses. The intercellular RNA was extracted and used as template for reverse transcription and 30 rounds of PCR amplification using the TAQ polymerase and different primer pairs. (A) PCR products spanning the hypervariable region of MHV-A59, JHM, and MHV-2 (642-2155) (lanes 2–4, respectively; lane 5, uninfected control); (B) Southern blot analysis of the PCR products shown in (A) using an internal oligomer probe; (C) PCR products spanning the hypervariable region in the S glycoprotein gene of MHV-A59 (lane 2) and RNA recombinant viruses derived from NC17 × NC16 (lanes 3–5), NC6 × LA7 (lanes 6–8), and NC16 × LA7 (lanes 10–12); (D) PCR products spanning the C (lanes 2–11) and N (lanes 12–21) termini of MHV-A59 (lanes 2, 12) and RNA recombinant viruses derived from NC17 × NC16 (lanes 3–5 and 13–15), NC6 × LA7 (lanes 6–8 and 16–18), and NC16 × LA7 (lanes 9–11 and 19–21); (E) PCR products spanning the hypervariable region of NC17 (lane 2) and four different revertant viruses (lanes 3–6). Lane 1 contains DNA markers in all panels.

polymerase region of MHV-A59 may be grossly underestimated because the exact location of the ts allele in the group E mutant (LA18) and group A mutants (LA3/LA6) was unclear. This seems unlikely because a uniform recombination frequency of 1%/575 nucleotides of dsRNA throughout the genome would: (1) predict

the recombination frequency for the MHV genome to approach ~56% or more; (2) place RNA$^-$ complementation groups A, B, and C in ORF 1b and RNA$^-$ complementation groups D and E in the p30/HE nonstructural proteins, and (3) suggest that conditional lethal mutants were not isolated within ORF1a at the 5′-most three-fifths (~14 kb) of the genome. This is highly unlikely since several proteolytic, hydrophobic membrane-anchoring and cysteine-rich domains have been identified in ORF 1a, which should be amenable to mutagenesis (Baker *et al.*, 1989; Lee *et al.*, 1991). More importantly, T1 fingerprint analysis of RNA recombinant viruses derived from ts mutants in complementation groups C, D, and E clearly place each genetic function in the polymerase gene (Keck *et al.*, 1987; Lai *et al.*, 1985). Since RNA$^-$ complementation groups C, D, and E have also been demonstrated to function in mRNA synthesis and map within ORF 1b, which contains polymerase, helicase, and metal-binding sequence motifs, it is extremely unlikely that these mutants map in the HE or p30 subgenomic ORFs (Baric *et al.*, 1990; Bredenbeck *et al.*, 1990; Lee *et al.*, 1991; Schaad *et al.*, 1990). In contrast, the recombination rate predicted through the polymerase region was not inordinately high (1%/1800–2500 bp of dsRNA), but rather closely approximated the recombination frequencies estimated to occur in apthovirus and poliovirus infections (Cooper, 1977; King, 1987). Thus, it seems likely that the frequencies measured between the polymerase and S glycoprotein genes actually represent true differences in the recombination rate.

Since the enhanced recombination frequencies in MHV were observed within a physically smaller (4.0-kb) region as compared to the 22-kb polymerase region, it is possible that a mechanism similar to high negative interference in DNA viruses may account for the increased rates of recombination (Chase and Doermann, 1958). This seems unlikely since high negative interference is probably mediated by DNA polymerase repair mechanisms, which have not been demonstrated in the MHV RNA polymerase (Glickman and Radman, 1980).

The increase in the recombination rate in the S glycoprotein gene could also not be attributed to a combination of true homologous recombination and recombination resulting in large deletions. Nonhomologous recombination probably explains the appearance of defective interfering RNAs of Sindbis virus, vesicular stomatitis virus, and MHV (Holland, 1987; Makino *et al.*, 1988, 1989; Monroe and Schlesinger, 1983). Since deletions have been demonstrated in the MHV S and HE glycoprotein genes as well as in the nonstructural genes encoded in mRNA 4 and 5 and in the TGEV S glycoprotein gene and other subgenomic ORFs (Banner *et al.*, 1990; La Monica *et al.*, 1991; Luytjes *et al.*, 1987; Parker *et al.*, 1990; Rasschaert *et al.*, 1990; Schwartz *et al.*, 1990; Yokomori and Lai, 1991), these data have suggested that the subgenomic ORFs may be very amenable to frequent deletions and insertions. Insertions in the MHV S glycoprotein gene have also been reported previously (Taguchi *et al.*, 1987). Analysis of 20 RNA recombinant viruses derived from different crosses between the group F mutants suggested that large deletions or insertions were rare and occurred at $< \frac{1}{20}$ the rate of true homologous recombination. While these data could not conclusively rule out the presence of very small deletions, this seems unlikely since the predicted distances between the ts alleles used in these crosses should have resulted in deletions ranging from ~100 bp to >1000 bp in length. It is also unlikely that the ts$^+$ virus isolated from these crosses were revertants containing small deletions (<50 bp) in a single ts parent, since the ratio of recombinants: revertants was at least 9:1 or greater in each of the crosses and none of the revertants analyzed from each parental ts mutant had evidence of deletions. Since revertants of SB ts mutants usually contain single nucleotide reversions at the site of mutation (Hahn *et al.*, 1989a,b), the most likely interpretation from these data is that the increase in the rate of recombination in the S glycoprotein gene probably reflects an increase in the rate of true homologous RNA recombination. If the mechanism for homologous recombination and recombinations resulting in deletions are similar as suggested by Banner *et al.*, 1990, these data suggest that the frequency of these two recombination events is very different. Alternatively, the regions flanked by the ts mutations used in these crosses may be critical for S glycoprotein gene function or plaque formation and could not be deleted.

Two possible mechanisms could explain variable rates of recombination in the MHV genome. The first possibility is a preferred recombination site in the MHV S glycoprotein gene. A clustering of RNA recombination sites adjacent to the hypervariable region in the S glycoprotein region suggest the presence of a preferred site of recombination (Banner *et al.*, 1990). However, in the absence of selection, crossover sites spanning the hypervariable region of the MHV S glycoprotein gene were random, suggesting that the preferred site of recombination reflects *in vitro* selection for certain types of recombinants (Banner and Lai, 1991).

Although the majority of data support non-site-specific homologous recombination throughout the entire poliovirus genome (Kirkegaard and Baltimore, 1986; Sarnow *et al.*, 1990), extensive computer analysis of 40 intertypic recombination sites in apthoviruses and poliovirus suggest that two thirds of all template

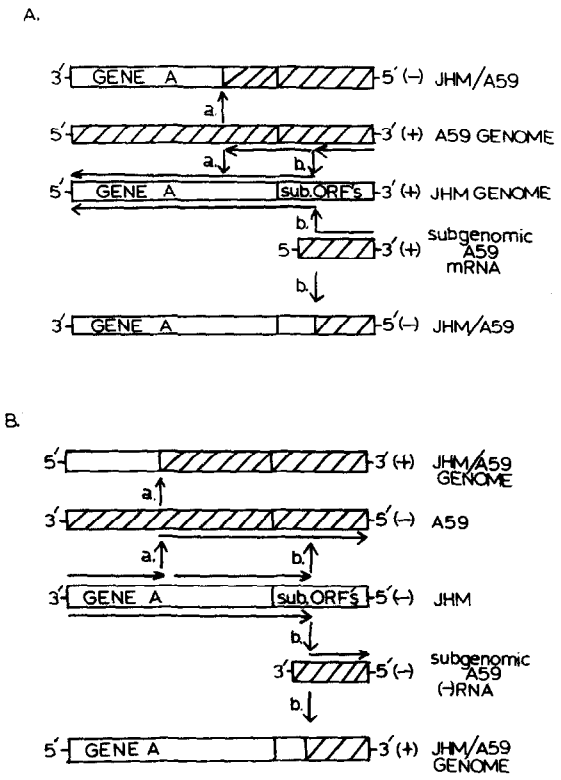

FIG. 7. Tentative model explaining variable rates of recombination in the MHV genome. Previous studies by Sethna and Brian, 1989, and Sawicki and Sawicki, 1990, have clearly demonstrated the presence of subgenomic minus strands in MHV and TGEV infected cells. If the subgenomic minus strands participate in template switching with full-length genomic RNAs, recombination rates should increase from the 5′ to the 3′ end of the genome. Panel A demonstrates how template switching could occur during negative-strand synthesis between a full-length and subgenomic-length plus strand RNA and result in a full-length negative-stranded RNA recombinant molecule. Panel B demonstrates how template switching between full-length and subgenomic negative strands during positive-strand synthesis could result in recombinant genome-length molecules.

switching occur after the synthesis of UU in sites chosen to minimize the adverse free energy change involved in switching to a heterotypic template (King, 1988). In addition, a nonrandom distribution of recombination sites has also been reported among intertypic poliovirus recombinants (Tolskaya *et al.*, 1987). If MHV RNA recombination is mediated by freely segregating RNA segments that are generated by transcriptional pausing during RNA synthesis, preferred sites of recombination may also exist in AU-rich regions and/or in regions of secondary structure in the MHV genome (Baric *et al.*, 1987). However, it is difficult to envision how these types of preferred sites of recombination would result in higher intratypic recombination frequencies between MHV-A59 ts mutants since extensive secondary structure is found throughout the MHV genome and the G:C:A:U and AA/UU dimer ratios are roughly equivalent in the polymerase and S glycoprotein regions (Banner *et al.*, 1991; Baric *et al.*, 1987; Soe *et al.*, 1988; Fu *et al.*, unpublished).

A more likely mechanism to explain the increase in the recombination rate in the S glycoprotein gene is based on the basic replication strategy of coronaviruses (Fig. 7). Recently, subgenomic minus-strand and replicative intermediate RNAs have been demonstrated during TGEV and MHV infection (Sethna *et al.*, 1989, 1991; Sawicki and Sawicki, 1990). These data indicate that the amount of negative-strand template RNA is unequal and increases from the 5′ to 3′ end of the genome. Because of the availability of more negative strand template, recombination rates should increase proportionately from the 5′ to 3′ end of the genome and be highest in the N gene coding region contained within mRNA 7. For example, recombination events in the polymerase region can only involve template switching between full-length negative- or positive-strand RNA templates. However, recombination events in the S glycoprotein gene or other subgenomic ORFs could not only occur between full-length RNA templates but also involve subgenomic mRNA and subgenomic negative-strand RNA templates as well.

Several findings support this hypothesis. First, recombination frequencies between the polymerase gene and S glycoprotein genes of MHV may vary threefold. Second, *in vitro* transcribed subgenomic-length DI RNAs rapidly undergo RNA recombination with full-length (or subgenomic) RNAs supporting the notion that recombination events can occur between different-sized template RNAs (Makino *et al.*, 1989). Third, analysis of sepharose 2B-CI column purified full-length RI RNA has demonstrated the presence of mRNA 7 subgenomic nascent-plus strands bound to the full-length negative-strand RNA (Baric *et al.*, 1983). While the original interpretation of these data supported "leader-primed" transcription, an alternative explanation for these findings is that these subgenomic nascent-plus strands have disassociated from subgenomic RI RNA template and recombined with the full-length negative-strand RNA. Finally, a large number of RNA recombinant viruses selected with markers in the S glycoprotein gene of MHV contain additional crossover sites in the M and N structural genes encoded at the 3′ end of the genome (Keck *et al.*, 1988b). These findings are consistent with the idea that subgenomic minus strands function in RNA recombination and suggest that recombination events may increase in frequency toward the 3′ end of the genome.

High-frequency RNA recombination is a unique property associated with MHV replication. Our data suggest that the frequency of RNA recombination is mediated in part by the large size of the MHV genome (32

kb) and its novel mechanism for RNA synthesis involving "leader primed" discontinuous transcription and mRNA replication via subgenomic minus strands (Baric *et al.,* 1983, 1987, 1990; Sethna *et al.,* 1989, 1991; Sawicki and Sawicki, 1990). The possibility that the MHV recombination rate increases proportionately from the 5′ to 3′ ends of the genome may also contribute to the evolution, genetic diversity, and organization of coronavirus genomes. For instance, the gene order of IBV is different from other coronaviruses and it contains an additional gene between the M and N genes at the 3′ end of the genome (Cavanagh *et al.,* 1991). TGEV also contains an additional gene, designated X, which is located at the 3′ end of the genome (Kapke and Brian, 1986). More remarkably, some coronaviruses, but not all, contain a p30 nonstructural protein and an HE glycoprotein that is related to the influenza C virus HE (Luytjes *et al.,* 1988). Coronaviruses and toroviruses may also have diverged by nonhomologous recombination events in their polymerase and envelope proteins (Snijder *et al.,* 1991). These findings are consistent with the fact that the coronavirus genome has a remarkable ability to evolve by homologous and nonhomologous recombination, especially in the genes encoded by subgenomic mRNA.

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