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Genetics of Mouse Hepatitis Virus Transcription: Identification of Cistrons which May Function in Positive and Negative Strand RNA Synthesis

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Received December 7, 1989; accepted April 20, 1990

A panel of 26 temperature-sensitive mutants of MHV-A59 were selected by mutagenesis with either 5-fluorouracil or 5-azacytidine. Complementation analysis revealed the presence of one RNA⁺ and five RNA⁻ complementation groups. None of the RNA⁻ complementation groups transcribed detectable levels of positive- or negative-stranded RNA at the restrictive temperature. Temperature shift experiments after the onset of mRNA synthesis revealed at least two classes of RNA⁻ mutants. RNA⁻ complementation groups A, B, D, and E were blocked in the ability to release infectious virus and transcribe mRNA and genome, while group C mutants continued to release infectious virus and transcribe both mRNA and genome. Temperature shift experiments at different times postinfection suggest that the group C mutants encode a function required early in viral transcription which affects the overall rate of positive strand synthesis. Analysis of steady state levels of negative strand RNA after the shift indicate that the group C mutants were probably blocked in the ability to synthesize additional minus strand RNA under conditions in which the group E mutants continued low levels of minus strand synthesis. These data suggest that at least four cistrons may be required for positive strand synthesis while the group C cistron functions during minus strand synthesis. © 1990 Academic Press, Inc.

INTRODUCTION

Mouse hepatitis virus (MHV), a member of Coronaviridae, contains a single-stranded, nonsegmented, plus-polarity RNA of 8.0×10^6 Da molecular weight. The 32-kb genomic RNA is organized into seven or eight genetic regions each encoding one or more viral proteins (Lai *et al.*, 1983; Pachuk *et al.*, 1989; Siddell, 1983). In the virion, the RNA is enclosed in a helical nucleocapsid structure constructed from multiple copies of a 50- to 60-kDa phosphorylated nucleocapsid protein (N) (Skinner and Siddell, 1983; Stohlmán *et al.*, 1988). The viral envelope is derived from modified host internal membranes and contains two virus-encoded glycoproteins designated M(gp23) and S(gp180/90) (Armstrong *et al.*, 1984; Shieh *et al.*, 1989; Sturman and Holmes, 1985; Sturman *et al.*, 1980). A third membrane glycoprotein, HE (gp65), has been identified in some strains of MHV (Luytjes *et al.*, 1988; Shieh *et al.*, 1989; Yokomori *et al.*, 1989).

Upon entry into the host cell, the genomic RNA is translated into a RNA-dependent RNA polymerase which directs the synthesis of a full-length negative-stranded RNA (Brayton *et al.*, 1982, 1984; Lai *et al.*, 1982). More recently, subgenomic minus strands were identified in MHV- and transmissible gastroenteritis coronavirus (TGEV)-infected cells (Sawicki and Sa-

wicki, 1990; Sethna *et al.*, 1989). In turn, the negative-stranded RNA acts as template for the synthesis of seven virus-specific mRNAs (Brayton *et al.*, 1982, 1984; Lai *et al.*, 1983; Leibowitz *et al.*, 1981). These RNAs are capped, polyadenylated, and arranged in the form of a nested set structure from the 3' end of the genome such that the sequences in each smaller RNA are contained within the next larger RNA species (Lai *et al.*, 1983, 1984). Each mRNA also contains a small ~72 nucleotide leader RNA at the 5' end which is derived from the 5' end of the genome (Lai *et al.*, 1984; Spaan *et al.*, 1983). Recent findings of subgenomic minus strands and replicative form (RF) RNA (Sawicki and Sawicki, 1990; Sethna *et al.*, 1989) in TGEV- and MHV-infected cells indicate that the exact mechanism for MHV transcription is unclear and requires additional study. In addition, little is known concerning the location and function of individual viral genes which participate in RNA synthesis. It is suspected that one or more viral proteins are encoded which regulate negative strand synthesis, leader RNA synthesis, mRNA synthesis, and genome replication (Spaan *et al.*, 1988). Genetic analysis of the MHV polymerase genes is needed to discern the nature of the viral polymerase and distinguish among the alternative mechanisms of RNA synthesis.

Temperature-sensitive (ts) mutants of animal viruses are useful for assigning particular physiologic, bio-

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chemical, and pathogenic functions to individual viral genes. Ts mutants for MHV have previously been reported and suggest that at least six RNA⁻ and two RNA⁺ complementation groups are encoded in the genome (Koolen *et al.*, 1983; Leibowitz *et al.*, 1982; Martin *et al.*, 1988); however, the role of these complementation groups in RNA synthesis has not been established. In this report, we describe the isolation and partial characterization of a panel of ts mutants of MHV-A59 which comprise one RNA⁺ and five RNA⁻ complementation groups. Temperature shift experiments after the onset of positive strand synthesis indicate that four of the RNA⁻ complementation groups (A, B, D, and E) are blocked in the ability to transcribe positive-stranded RNA. Conversely, group C mutants are probably defective in the synthesis of negative-stranded RNA, but not mRNA or genome. Genetic recombination analysis indicates that the five RNA⁻ complementation groups are encoded in a linear array at the 5' end of the genome (Baric *et al.*, 1990).

MATERIALS AND METHODS

Virus and cell lines

The A59 strain of mouse hepatitis virus (MHV-A59) was used throughout the course of this study. Viruses were propagated and cloned three times in the continuous murine astrocytoma cell line, DBT, or in L2 cells (Lai *et al.*, 1985). Prior to use in these experiments, cloned MHV-A59 at passage level six was plaque purified three times in DBT cells at 39° and virus stocks were prepared. DBT or L2 cells were grown in Dulbecco's modified minimum essential medium (DMEM) containing 10% Nu-serum (Irvine Scientific) or 10% fetal calf serum (FCS) (Hyclone) supplemented with 100 U/ml penicillin G/amphotericin B (0.25 µg/ml). Virus stocks were prepared on DBT cells containing 5% FCS. All plaque assays were performed on monolayers of DBT cells in DMEM containing 5% Nu-serum, 0.8% agarose, and 100 U/ml penicillin G/amphotericin B (0.25 µg/ml). Plates were incubated at 32 or 39.5° for 30–48 hr, and plaques visualized with neutral red.

Mutagenesis

L2 or DBT cells were infected with MHV-A59 at a m.o.i. of 10 at 32°. Two hr postinfection the medium was removed and replaced with DMEM containing 2% FCS and either 20 µg/ml 5-azacytidine or concentrations of 350 or 450 µg/ml 5-fluorouracil. These concentrations were determined to inhibit virus multiplication by approximately 90% under these conditions of treatment. Mutagenized-virus progeny were harvested at 16 hr postinfection and stored at -70°. Alternatively, cultures of cells were infected with MHV-A59 for 1 hr

and overlaid with medium containing 30 µg/ml 5-azacytidine or concentrations of 75 or 100 µg/ml 5-fluorouracil. Virus titers were also reduced by approximately 90% under these alternative treatment conditions. A total of seven different mutagenized stocks were prepared to ensure that ts mutants with unique mutation sites would be represented with each complementation group of MHV-A59. Independently isolated mutations were necessary for the formulation of an accurate recombination map (Baric *et al.*, 1990).

Selection of mutants

Stocks of mutagenized virus were plaque assayed at 32° (permissive temperature). After 36–48 hr incubation, individual plaques were removed, diluted 1/100 in PBS, plated onto monolayers of DBT cells on gridded plates, and overlaid with DMEM containing 0.8% agarose and 2% FCS. Virus isolates which formed plaques after 36–72 hr at permissive but not nonpermissive temperature (39.5°) were repurified and propagated on cells for future use. The reversion frequency of each ts mutant was determined at 39.5°/32° by plaque assay on DBT cells and only those which had differential titers of 10⁻³ were retained for further study.

Determination of RNA phenotype

The ability of the mutants to synthesize RNA at the nonpermissive temperature was determined by infecting monolayers of DBT cells in 24 well culture plates (Falcon Plastics, Oxnard, CA) at a m.o.i. of 5. Following adsorption for 2 hr at 32°, the inoculum was removed and DMEM containing 2% FCS and 2 µg/ml actinomycin D was added to inhibit cellular transcription. The plates were incubated at 39.5° until 6 hr postinfection when 1 µCi/ml of [³H]uridine (ICN) was added. Under these conditions, viral but not host RNA is radiolabeled (Lai and Stohlman, 1978). Following two additional hours of incubation, the medium was removed and the monolayers were solubilized with 1.0% SDS in water. Four replicas per sample were analyzed for evidence of virus-directed RNA synthesis and the average was determined by precipitation with the addition of an equal volume of 10% trichloroacetic acid. The incorporation of [³H]uridine into precipitate counts was determined by filtration onto Whatman GFA filters. Controls included wild-type MHV-A59-infected and uninfected controls treated with 2 µg/ml actinomycin D.

Complementation

Complementation analyses of the RNA⁻ and RNA⁺ ts mutants were performed in quadruplicate on DBT monolayers in 24-well plates co-infected with ts mutants at a m.o.i. of approximately 10 each. Following adsorption for 1 hr at 32°, the inoculum was removed,

the monolayers were washed with 2 ml of PBS, and the plates were incubated in 2 ml of complete medium at 39.5°. To help reduce background and increase the sensitivity of the assay, medium containing unadsorbed virus was removed at 2 hr postinfection and replaced with complete medium prewarmed to 39.5°. Supernatants were harvested at 16 hr postinfection and compared with the virus titer from singly infected cultures at 39.5° by plaque assay at 32 and 39.5°. The ratios of these two numbers were used to compute the complementation index (CI) as described by the following formula:

$$CI = \frac{(A \times B)_{32^\circ} - (A \times B)_{39.5^\circ}}{(A + B)_{32^\circ}}$$

A and B represent the two ts mutants being crossed at 39.5° for complementation analysis. The progeny were plaque assayed at either 32 or 39.5°. A CI \geq 3 between any pair of mutants in at least three different assays was considered positive for complementation. Conversely, mutants exhibiting a CI \leq 3 were considered to have defects in the same genetic function and grouped accordingly (Leibowitz *et al.*, 1982). RNA⁻ mutants which complemented each other's genetic defect produced syncytia and giant cell formation within 12 hr postinfection.

Detection of MHV intracellular RNA

Duplicate cultures of DBT cells were infected with different ts mutants at a m.o.i. of 4 for 1 hr at room temperature. Medium was added and one-half of the cultures were incubated at either 32° or 39.5° for 8 hr. Intracellular RNA was extracted by the following protocol. DBT cells were rinsed and pelleted in ISO-TKM buffer (10 mM Tris-HCl, pH 7.5, 150 mM KCl, and 1.5 mM MgCl₂). After 5 min centrifugation at 1000 rpm, the pellets were resuspended in a solution containing 5 vol Hypo-TKM buffer (10 mM Tris-HCl, pH 7.5, 10 mM KCl, and 1.5 mM MgCl₂) and NP-40 to a final concentration of 0.5%. The nuclei were pelleted and the supernatants extracted with an equal volume of ultrapure phenol (U.S. Biochemical Co.) and SDS to a final concentration of 0.5%. The supernatants were then extracted with an equal volume of phenol/chloroform followed by precipitation in 2 or 3 vol of 95% ethanol.

Equivalent amounts of extracted RNA were adsorbed to nitrocellulose filters and hybridized with strand-specific RNA probes which detected MHV minus strand RNA, mRNA, or genome. A *StyI/PstI* restriction fragment approximately 1.6 kb in size from cDNA clone A9 was subcloned into an IBI T7/SP6 transcription vector in the positive (IBI20N) or negative (IBI76N) orientation. The A9 clone represents JHM sequences 693 to ~2370 encoding the 3'-most portion of the E1

gene (mRNA 6), the intergenic sequences, and the entire N gene (mRNA 7) inserted into the vector pBR322. The exact 3' terminus of A9 is unclear because of the lack of sequence data but has been approximated by size analysis and restriction mapping. Utilizing the T7 promoter, transcription of IBI76N results in a negative-sensed transcript which detects both subgenomic mRNAs and genomic RNA. Conversely, T7 transcription of IBI20N results in a positive-sensed transcript which will detect full length as well as subgenomic-length negative-sensed RNA; however, this probe will not allow us to distinguish between the two. To specifically detect the presence of genomic RNA, but not mRNA, a negative-sensed RNA transcript from clone 5F82⁻ (nucleotides 5-1149 at the 5' end of the genome) (Baric *et al.*, 1988; Stohman *et al.*, 1988) was synthesized *in vitro*. For some experiments, in an attempt to improve the sensitivity of our probes, T7 transcripts were also synthesized from clone 5F82⁺ (as described above, but in the positive orientation) and mixed with IBI20 transcripts to detect negative-stranded RNA.

The ³²P-radiolabeled RNA probes were synthesized *in vitro* as previously described and 2 × 10⁶ cpm/ml hybridized to RNA bound to 0.45 μm nitrocellulose filters in 50% formamide, 5× SSC, 10 mM Na phosphate (pH 6.5), 5× Denhardt's, 125 μg/ml salmon sperm DNA, 125 μg/ml tRNA, and 8.3 mM EDTA at 65° for 24 hr (Baric *et al.*, 1988). Filters were washed and exposed to XAR-5 film in the presence of an intensifying screen.

Virus growth curves and RNA synthesis following temperature shift

Cultures of DBT cells, grown in 100-mm dishes, were infected at a m.o.i. of 4 with different ts mutants and maintained at 32°. Following incubation at 32° for 5–6 hr, duplicate cultures were shifted to restrictive temperature by the addition of prewarmed media and virus progeny harvested at varying intervals for analysis by plaque assay. In addition, intracellular RNA was extracted at similar times postinfection and analyzed for the presence of viral mRNA with strand-specific RNA probes which detect mRNA, genome, or negative-stranded RNA as previously described. Filters were washed four times with 2× SSC containing 0.01% SDS and four times with 0.2× SSC containing 0.1% SDS at 75°. The filters were exposed to XAR-5 film with an intensifying screen, and the bands were localized, excised, and counted. Duplicate blots were also probed with an actin cDNA probe to demonstrate that equivalent amounts of RNA were bound to the filters at different times postinfection.

To examine rates of mRNA synthesis, cultures of cells were also infected with different mutants and maintained at 32° for 3 hr. The cultures were treated

with 2 $\mu\text{g/ml}$ actinomycin D and shifted to 39.5° by the addition of prewarmed media at various times postinfection. At 7.5 hr postinfection, the cultures were radio-labeled with 100 $\mu\text{Ci/ml}$ [^{32}P]orthophosphate for 1.5 hr. Intracellular RNA was extracted, treated with glyoxal and separated on 1% agarose gels. [^{32}P]Orthophosphate rather than [^3H]uridine was used in these studies to circumvent high levels of poly(U) polymerase activity previously detected in MHV-infected cells (Compton *et al.*, 1987). Viral bands were visualized by exposure to XAR-5 film with an intensifying screen.

RESULTS

Isolation and phenotypic characterization of the ts mutants

Mutants designated LA were isolated in Los Angeles while those designated NC were isolated in North Carolina. To help ensure that unique mutation sites were present within our panel, several different mutagenesis experiments were performed (as described under Materials and Methods). The results from these assays are shown in Table 1. Eleven ts mutants were isolated from MHV-A59-infected cells following treatment with 20 $\mu\text{g/ml}$ 5-azacytidine. Five additional mutants were isolated from cultures treated with 30 $\mu\text{g/ml}$ of the drug. Ten mutants were isolated from 5-fluorouracil treatment by the addition of the drug at Time 0 (75 or 100 $\mu\text{g/ml}$) or at 2 hr postinfection (350 or 450 $\mu\text{g/ml}$). All the mutants retained after the initial screening had reversion frequencies ranging from 9.5×10^{-3} to 1.0×10^{-6} .

The ability of each mutant to synthesize virus-specific RNA at the nonpermissive temperature was determined by the incorporation of the [^3H]uridine into acid precipitate material from actinomycin D-treated virus-infected cells. In Table 1, the ability of each ts mutant to synthesize viral RNA is shown as a percentage of the wild-type-infected controls. Six of the ts mutants (LA7, LA12, LA13, NC5, NC6, and NC7) were considered to be of the RNA positive phenotype since levels of transcription at 39.5° was $\geq 38\%$ of the wild-type virus. The remaining 20 mutants were of the RNA⁻ phenotype since transcription at 39.5° was $\leq 8\%$ of the parental virus. The frequency of RNA⁺ to RNA⁻ mutants in this panel was comparable to those previously described (Koolen *et al.*, 1983; Leibowitz *et al.*, 1982; Martin *et al.*, 1988).

Complementation analysis of MHV-A59 ts mutants

Complementation analysis of viral mutants is an effective method for determining the number of genes or genetic functions encoded by a virus. To determine the number of genetic functions which participate in

viral RNA transcription, we analyzed the RNA⁻ and RNA⁺ ts mutants by complementation analysis. DBT cells were infected with different mutants either individually or in combination and incubated at 39.5° for 16 hr. Cells infected with mutants capable of complementing each others genetic functions were characterized by syncytia formation, the characteristic CPE induced in susceptible cells. Complementation indexes for the individual crosses are shown in Table 2 and summarized in Table 3. These data indicate that one RNA⁺ and five RNA⁻ complementation groups are included within this panel of mutants. The groups were designated A through F according to their order from the 5' end of the genome by genetic recombination mapping (Baric *et al.*, 1990). One mutant (LA16) was determined to be a double mutant in the group A and group B allele.

Viral RNA synthesis at nonpermissive temperature

The majority of MHV-A59 ts mutants were incapable of transcribing RNA at the nonpermissive temperature (Table 1). However, this approach is not sensitive enough to detect the synthesis of small quantities of negative-stranded RNA. To determine if minus strand RNA synthesis occurs at nonpermissive temperature, cells were infected with different ts mutants at a m.o.i. of 4 at 39.5° and intracellular RNA harvested 8 hr postinfection. The RNA was denatured, bound to nitrocellulose filters, and probed with strand-specific RNA probes for the presence of either mRNA (IBI76N), or negative strand RNA (IBI20N and 5F82⁺). This approach will detect steady state levels of negative strand RNA synthesis between 1 and 8 hr postinfection. None of the RNA⁻ complementation groups transcribed detectable levels of either mRNA (Fig. 1A) or negative-stranded RNA (Fig. 1B) under these conditions of treatment. Conversely, the RNA⁺ mutants and wild-type MHV-A59 synthesized both positive and negative RNA at restrictive temperature. It is unclear why the RNA⁺ mutants transcribe reduced amounts of mRNA at restrictive temperature as compared to the MHV A59 control. However, these data are consistent with the labeling experiments shown in Table 1. Under these conditions, the data suggest that the RNA⁻ mutants are defective in positive and negative RNA synthesis when maintained at the restrictive temperature. The possibility remains, however, that by 8 hr postinfection minus strand RNA may have been synthesized and degraded.

Virus growth curves following temperature shift

The basic genetics of alphavirus transcription and replication have been elucidated by temperature shift experiments immediately after the onset of positive strand RNA synthesis (Sawicki *et al.*, 1981a,b, 1978).

TABLE 1
TEMPERATURE-SENSITIVE MUTANTS OF MHV-A59

Mutagen designation	Titer (PFU/ml)		Reversion frequency 39°/32°	% RNA ^a	RNA phenotype	
	32°	39°				
5-Azacytidine (20 µg/ml)						
1	LA3	1.5 × 10 ⁶	1.0 × 10 ²	1.5 × 10 ⁻⁶	6.9	-
2	LA6	4.0 × 10 ²	1.0 × 10 ²	2.5 × 10 ⁻⁴	0.0	-
3	LA7	1.5 × 10 ⁶	1.1 × 10 ³	7.3 × 10 ⁻⁴	67.0	+
4	LA8	7.7 × 10 ⁶	1.4 × 10 ²	1.8 × 10 ⁻⁵	0.0	-
5	LA9	2.3 × 10 ⁵	3.3 × 10 ⁰	1.4 × 10 ⁻⁵	0.0	-
6	LA10	1.9 × 10 ⁶	1.6 × 10 ²	8.4 × 10 ⁻⁵	0.0	-
7	LA11	3.9 × 10 ⁶	1.0 × 10 ²	2.6 × 10 ⁻⁵	2.0	-
8	LA12	1.5 × 10 ⁶	ND	<1.5 × 10 ⁻⁶	44.0	+
9	LA13	6.5 × 10 ⁵	ND	<6.5 × 10 ⁻⁵	39.0	+
10	NC6	5.5 × 10 ⁷	1.3 × 10 ²	2.3 × 10 ⁻⁶	40.0	+
11	NC7	1.3 × 10 ⁸	1.1 × 10 ⁴	8.6 × 10 ⁻⁵	38.0	+
5-Azacytidine (30 µg/ml)						
12	NC1	5.3 × 10 ⁶	5.2 × 10 ³	5.5 × 10 ⁻⁶	7.5	-
13	NC2	5.6 × 10 ⁷	3.1 × 10 ³	5.5 × 10 ⁻⁵	1.0	-
14	NC3	2.2 × 10 ⁷	4.0 × 10 ³	1.8 × 10 ⁻⁴	0.0	-
15	NC4	1.2 × 10 ⁷	2.2 × 10 ²	1.8 × 10 ⁻⁵	0.0	-
16	NC5	1.5 × 10 ⁶	2.0 × 10 ²	1.3 × 10 ⁻⁴	57.0	+
5-Fluorouracil (75 µg/ml)						
17	NC13	9.0 × 10 ⁷	3.0 × 10 ³	3.3 × 10 ⁻⁵	8.0	-
5-Fluorouracil (100 µg/ml)						
18	NC8	2.7 × 10 ⁷	4.5 × 10 ²	1.6 × 10 ⁻⁶	0.0	-
19	NC9	1.5 × 10 ⁷	5.0 × 10 ¹	3.7 × 10 ⁻⁶	0.0	-
20	NC10	9.25 × 10 ⁷	1.0 × 10 ²	1.0 × 10 ⁻⁴	5.0	-
21	NC11	2.3 × 10 ⁸	2.5 × 10 ¹	1.0 × 10 ⁻⁷	3.0	-
22	NC12	5.8 × 10 ⁷	6.0 × 10 ³	1.0 × 10 ⁻⁴	1.0	-
5-Fluorouracil (350 µg/ml)						
23	LA14	1.7 × 10 ⁶	4.0 × 10 ¹	2.3 × 10 ⁻⁵	0.0	-
24	LA15	6.0 × 10 ⁵	ND	<6.0 × 10 ⁻⁵	2.0	-
5-Fluorouracil (450 µg/ml)						
25	LA16	4.9 × 10 ⁷	2.7 × 10 ⁴	5.5 × 10 ⁻⁴	4.0	-
26	LA18	1.3 × 10 ⁶	ND	<1.3 × 10 ⁻⁶	0.0	-

Note. ND, not detected; +/−, Positive or negative by Northern blot analysis.

^a Calculated as ts cpm 39.5°/wt MHV-A59 cpm at 39.5° × 100%.

To determine if temperature shift studies could distinguish between different functions in MHV RNA synthesis, cells were infected with mutants representing each complementation group and maintained at 32° for 5.5 hr. One-half of the cultures were then shifted to restrictive temperature by the addition of prewarmed medium and virus progeny were isolated at different times postinfection. Shift-up to the restrictive temperature blocked the release of infectious virus from RNA⁻ com-

plementation groups A, B, D, and E and from the RNA⁺ group F. In contrast, complementation group C (RNA⁻) continued to release infectious virus after shift to restrictive temperature (Fig. 2).

Detection of positive strand RNA synthesis following temperature shift

The release of infectious virus following temperature shift indicated the presence of two classes of RNA⁻

TABLE 2

COMPLEMENTATION INDEX (CI) OF MHV RNA⁻ ts MUTANTS

Group	Mutant	LA6	LA16	LA3	NC2	LA9	LA14	LA10	LA18	LA7
A	LA6	—	<1	3.1	ND	17.0	21.0	120.3	25.7	ND
	LA16	—	—	<1	<1	395.0	7.0	180.0	91.3	8.8
	LA3	—	—	—	4.0	24.0	12.0	340.0	129.7	19.3
B	NC2	—	—	—	—	18.0	ND	11.0	12.0	4.5
C	LA9	—	—	—	—	—	<1	389.0	279.0	34.2
	LA14	—	—	—	—	—	—	55.0	5.0	27.0
D	LA10	—	—	—	—	—	—	—	26.5	22.0
E	LA18	—	—	—	—	—	—	—	—	24.0
F	LA7	—	—	—	—	—	—	—	—	—

mutants, one defective in an early function (group C) and one in a late function (groups A, B, D, and E). We next examined the ability of mutants from each complementation group to transcribe mRNA following shift to restrictive temperature. Duplicate cultures were infected with each mutant and maintained at 32° for 5–6 hr. One-half of the cultures were shifted to the restrictive temperature by the addition of prewarmed medium, and intracellular RNA was isolated at different times postinfection. RNA was then bound to nitrocellulose filters and probed with strand-specific RNA probes which detect mRNA and genome. Consistent with the inability to release infectious virus, RNA⁻ complementation groups A, B, D, and E were defective in mRNA and genome synthesis following shift to restrictive temperature. In contrast, the group C RNA⁻ mutant LA9 and group F RNA⁺ mutant LA7 continued mRNA and genome synthesis (Fig. 3). To confirm that the group C mutants were blocked in an early stage in mRNA synthesis, cells were infected with LA9 and shifted to restrictive temperature at 0, 3.5, 4.5, 5.5, and 6.5 hr postinfection. At 7.5 hr postinfection, 100 μ Ci/ml [³²P]orthophosphate was added for 1.5 hr. Intracellular RNA was extracted at 9.0 hr postinfection and separated on 1% agarose gels. Rates of LA9 (group C) mRNA synthesis

increased proportionately in cultures that were shifted to restrictive temperature at times later postinfection (Fig. 4A), and were reduced as compared to controls maintained at permissive temperature (Fig. 4B). In agreement with previous data, groups D and E were blocked in the ability to transcribe mRNA following shift to the restrictive temperature (Fig. 4C). These data indicate that the group C mutants are defective in an early component required for mRNA synthesis later in infection.

Negative strand synthesis following shift to restrictive temperature with the group C and E mutants

To distinguish between a viral function required during mRNA synthesis and a function required during

TABLE 3

COMPLEMENTATION GROUPS OF MHV-A59

Complementation group	Mutants	RNA phenotype
A	LA3, LA6, LA16, NC8, NC9, NC13	(-)
B	LA16, NC2, NC11	(-)
C	LA8, LA9, LA14, NC1, NC3, NC10	(-)
D	LA10	(-)
E	LA18, NC4, NC12	(-)
F	LA7, LA12, LA13, NC5, NC6	(+)

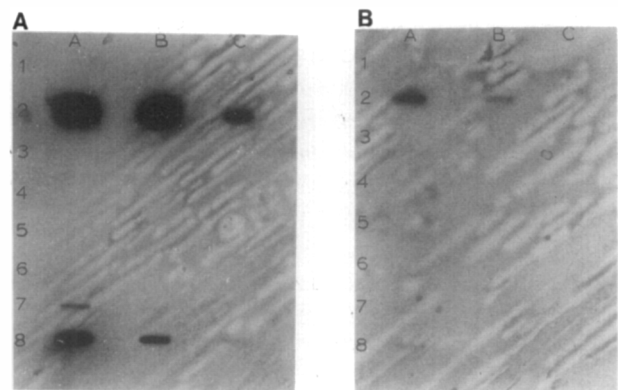


Fig. 1. Viral RNA synthesis at restrictive temperature. Cultures of cells were infected with ts mutants representing different complementation groups and maintained at 39.5° for 8 hr. The RNA was isolated, bound to nitrocellulose filters, and probed with strand-specific RNA probes that detect mRNA (A) or negative strand RNA (B). Lanes A, B, and C represent 10, 1, and 0.1 μ g RNA loaded per slot, respectively. Lanes 1 through 8: uninfected, MHV-A59, NC1 (group C), NC2 (group B), NC3 (group A), NC4 (group E), NC5 (group F), and LA7 (group F).

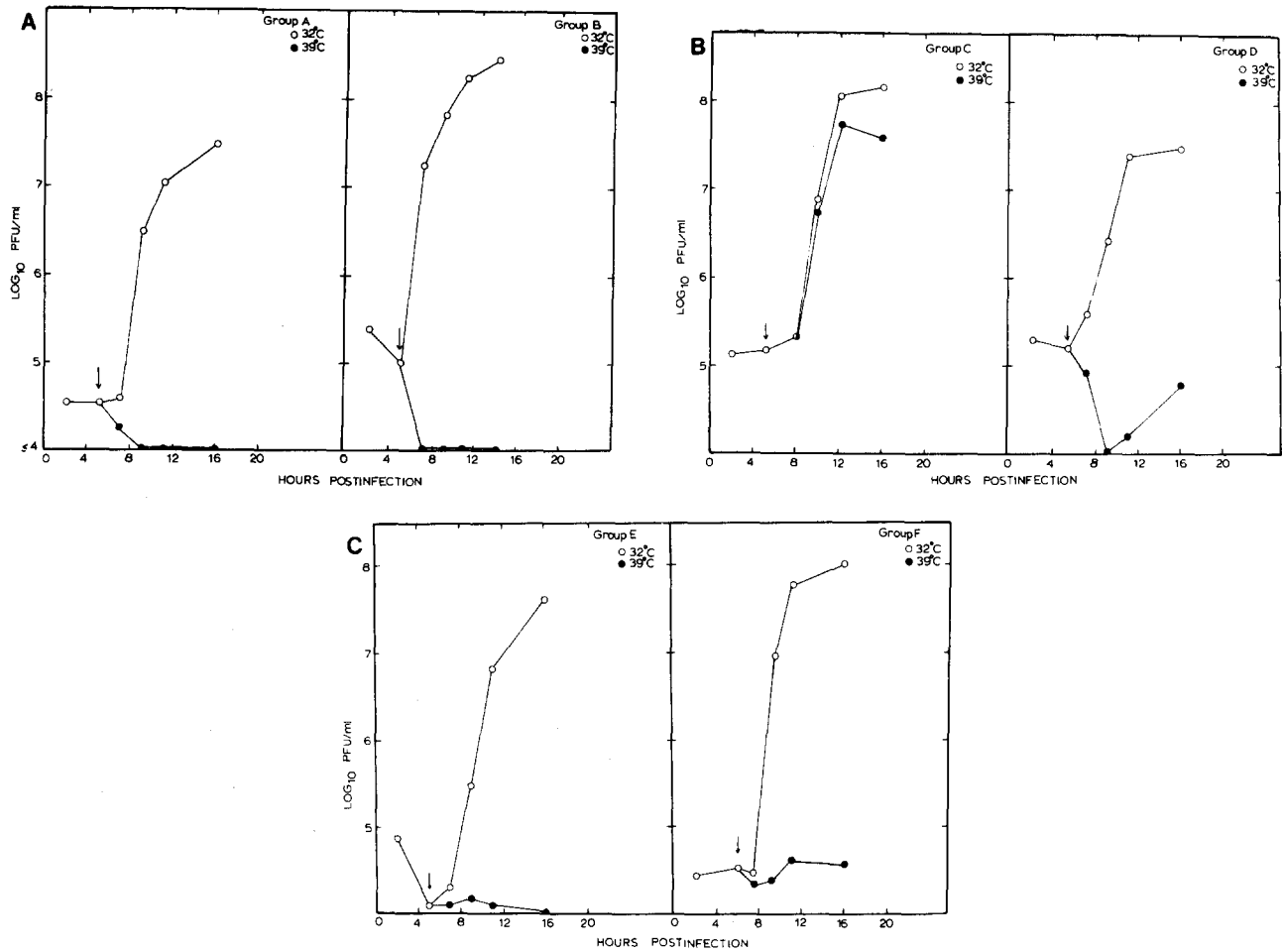


Fig. 2. Virus growth curves following shift to restrictive temperature. Duplicate cultures of cells were infected with different ts mutants representing each MHV complementation group at a m.o.i. of 4 and incubated at permissive temperature for 5 to 6 hr. One-half of the cultures were shifted to restrictive temperature by the addition of prewarmed medium. (A) Complementation groups A (LA3) and B (NC2); (B) Complementation groups C (LA9) and D (LA10); (C) Complementation groups E (LA18) and F (LA7). Symbols: ○, 32°; ●, 39.5°.

negative strand synthesis, we examined steady state levels of negative-stranded RNA synthesis following shift to restrictive temperature. Previous studies indicate that SP6-generated RNA transcripts are only 99.8% specific (Melton *et al.*, 1984). To demonstrate the specificity of our detection methods, genomic RNA from gradient-purified virions, and intracellular RNA from infected and uninfected cells, were bound to nitrocellulose filters and hybridized to radiolabeled RNA probes specific for viral mRNA (Fig. 5A) or negative strand RNA (Fig. 5B). Under conditions of excess genomic RNA, no cross-hybridization was detected with the probe that detects negative strand RNA and were in agreement with previous findings from our laboratory (Baric *et al.*, 1988).

Cultures of cells were infected with group C or group E mutants and shifted to the restrictive temperature at 5.5 hr postinfection. These conditions were chosen since viral mRNA synthesis has begun but not yet peaked under our treatment conditions at 32° (Fig. 4).

In addition, previous studies indicate that at high m.o.i. (50) at 37°, negative strand synthesis peaks at 6 hr postinfection (Sawicki and Sawicki, 1986). Intracellular RNA was isolated at different times postshift, bound to nitrocellulose filters, and probed for the presence of mRNA or negative strand RNA. In agreement with previous findings, viral mRNA transcription continues after shift with the group C but not group E mutants (Figs. 6A and 6B). At the permissive temperature (32°), steady state levels of group C and E negative-stranded RNA increased and peaked between 7 and 9 hr postinfection (Figs. 6C and 6D). Conversely, after shift to the restrictive temperature at 5.5 hr, we could not detect any increase in the amount of negative-stranded RNA with the group C mutants (Fig. 6C). Under identical treatment conditions, group E minus strand RNA levels continued to increase after shift and peaked between 7 and 9 hr postinfection. At the restrictive temperature, total levels of negative strand RNA were about 50% of the controls maintained at 32°. The different intensities

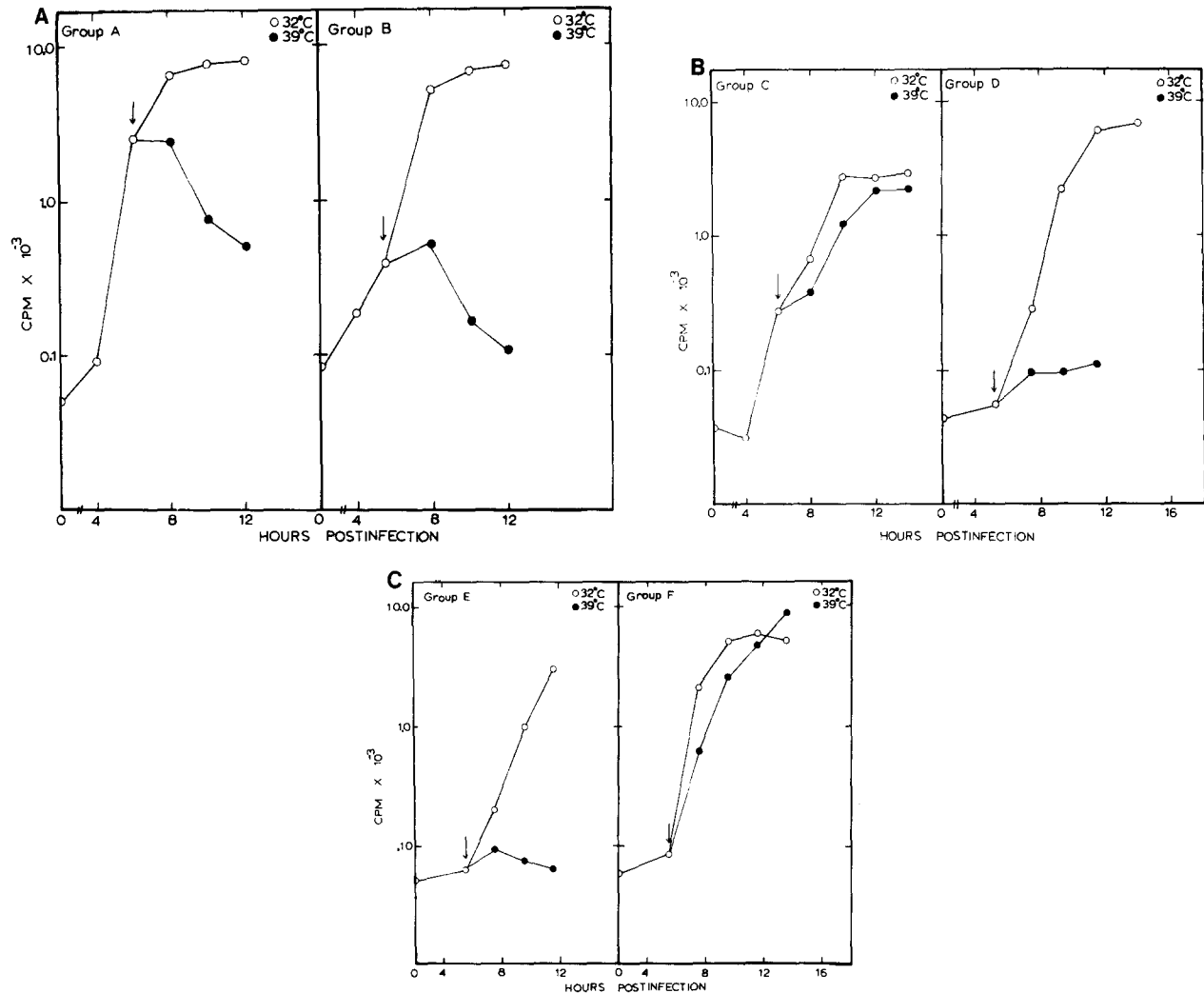


FIG. 3. Analysis of mRNA synthesis following shift to restrictive temperature. Duplicate cultures of cells were treated as described in the legend to Fig. 2. The extracted RNA was bound to nitrocellulose filters and hybridized with plus strand-specific mRNA probes. The filters were washed and exposed to XAR-5 film. Bands were visualized, excised, and counted. (A) Complementation groups A (LA3) and B (NC2); (B) Complementation groups C (LA9) and D (LA10); (C) Complementation groups E (LA18) and F (LA7). Symbols: O, 32°; ●, 39.5°.

of signal in the minus strand blots reflect different exposure times. Negative strand synthesis also continued to increase with the group F RNA⁺ mutants after shift (data not shown). These data are consistent with the interpretation that the group C allele probably functions during negative strand synthesis.

DISCUSSION

The ~32-kb MHV genome is divided into seven or eight coding regions which are translated into one or more viral proteins (Lai *et al.*, 1983; Luytjes *et al.*, 1988; Shieh *et al.*, 1989; Siddell, 1983). Analogous to other plus polarity RNA viruses, purified MHV genome is infectious suggesting that the polymerase genes are encoded at the 5' end of the genome (Lai and Stohman, 1978; Wege *et al.*, 1978). The ~23-kb putative poly-

merase gene of MHV is encoded in region A and has the capacity to encode 700–800 kDa of protein (Baker *et al.*, 1989; Pachuk *et al.*, 1989). Sequencing of the entire genome of another coronavirus, avian infectious bronchitis virus (IBV), suggests that the polymerase gene is composed of two large ORFs (F1 and F2) which potentially encode two polypeptides of 441 and 300 kDa, respectively (Bourne *et al.*, 1987). These ORFs are probably translated into a single large polyprotein by a ribosomal frame-shifting mechanism and then processed into several functional proteins (Brierly *et al.*, 1987). MHV genomic RNA is translated into a ~250-kDa polyprotein and subsequently cleaved into p28 and p220 proteins (Denison and Perlman, 1986, 1987; Soe *et al.*, 1987). An autoproteolytic activity has been demonstrated in the protein region corresponding to 3.9–5.3 kb from the 5' end of the genome (Baker *et al.*,

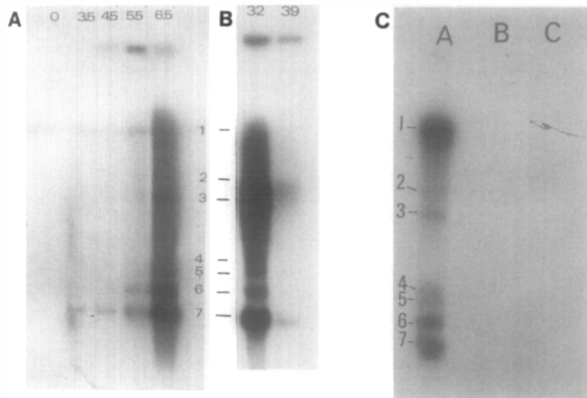


FIG. 4. Effect of temperature shift of group C mRNA synthesis. Cultures of cells were infected with the group C mutant LA9, shifted to the restrictive temperature at the indicated times, and radiolabeled with $100 \mu\text{Ci/ml}$ [^{32}P]orthophosphate for 1.5 hr at 7.5 hr postinfection. The radiolabeled nucleic acids were treated with glyoxal and separated on 1% agarose gels. (A) Group C mutant LA9 shifted to the restrictive temperature at the indicated times; (B) Group C mutant LA9 maintained at 32° or shifted to the restrictive temperature (39.5°) at 4.5 hr; (C) Lane 1: group E mutant LA18 at 32° ; Lane 2: group E mutant LA18 shifted to 39.5° at 5.5 hr; Lane 3: group D mutant LA10 shifted to restrictive temperature at 5.5 hr.

1989). Unfortunately, little information is available concerning the number, location, and function of the polymerase genes encoded in region A of either IBV or MHV.

Although the MHV polymerase(s) is poorly characterized, it must possess an array of activities designed to initiate and transcribe negative-stranded RNA, genomic RNA, mRNA, and leader RNA (Baric *et al.*, 1983, 1985, 1987; Spaan *et al.*, 1988). Additionally, the polymerase must possess the capacity to switch templates during transcription and promote high frequency recombination (Baric *et al.*, 1990; Keck *et al.*, 1987; Lai *et al.*, 1985; Makino *et al.*, 1986a). Previous studies have shown that continuous protein synthesis is required for

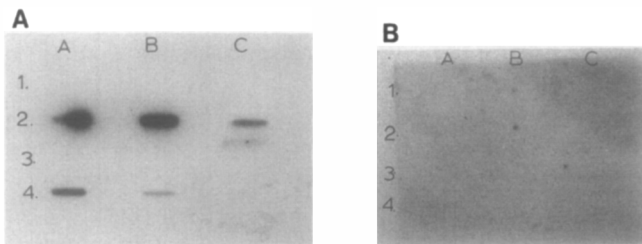


FIG. 5. Specificity of the strand-specific RNA probes. Viral RNA was extracted from gradient-purified virions or from virus-infected cells and bound to nitrocellulose filters as previously described. The filters were hybridized with strand-specific RNA probes which detect either positive or negative strand RNA. Lanes 1 through 4: uninfected, A59 genome, blank, infected intracellular RNA. (A) Probed for the presence of viral mRNA; (B) Probed for the presence of negative strand RNA. Lanes A, B, and C: 1, 0.1, and $0.01 \mu\text{g}$ RNA, respectively.

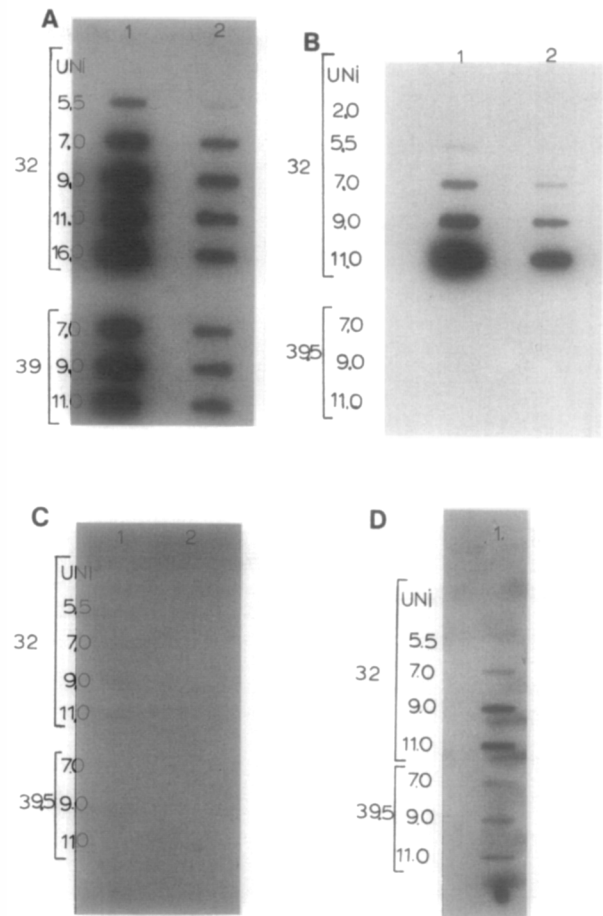


FIG. 6. Negative strand synthesis following shift to restrictive temperature. Duplicate cultures of cells were infected with complementation group C mutant LA9 or group E mutant LA18, and maintained at the permissive temperature for 5.5 hr. One-half of the cultures were shifted to the restrictive temperature and intracellular RNA isolated at various times postinfection. The RNA was bound to nitrocellulose filters and probed with strand-specific RNA probes that detect either MHV negative or positive strand RNA. The numbers along the Y axis represent the times postinfection intracellular RNA was extracted and the temperature. (A) Group C mutant LA9 probed for the presence of viral mRNA; (B) Group E mutant LA18 probed for the presence of viral mRNA. Lanes 1 and 2: 10 and $1 \mu\text{g}$ RNA, respectively; (C) Group C mutant LA9 probed for the presence of negative strand RNA. Lane 1: $5 \mu\text{g}$ RNA; lane 2: $0.5 \mu\text{g}$ RNA; (D) Group E mutant LA18 probed for the presence of negative strand RNA; $1 \mu\text{g}$ of RNA was bound to nitrocellulose.

positive and negative strand synthesis, and that temporal regulation of MHV negative strand synthesis and bovine coronavirus (BCV) genome replication occur during infection (Keck *et al.*, 1988a; Sawicki and Sawicki, 1986). Three polymerase activities have been described in *in vitro* transcription assays that function in the synthesis of negative strand RNA, genome length RNA, and mRNA. The early (1 hr postinfection) *in vitro* polymerase complex is thought to function in the synthesis of negative strand RNA (Brayton *et al.*, 1982, 1984). The MHV N protein is also associated

with the transcription complex, since anti-N monoclonal Abs block *in vitro* transcription, and an N binding site is present within the leader RNA sequence (Baric *et al.*, 1988; Compton *et al.*, 1987; Stohlman *et al.*, 1988). These data suggest that several distinct polymerase activities must function either separately or in concert to promote virus RNA synthesis.

The mechanism for MHV transcription is unclear. Recent findings of subgenomic minus strands and replicative form RNAs during coronavirus transcription warrant a reexamination of the leader-primed model of transcription (Baric *et al.*, 1983). Several alternative or modified mechanisms have been proposed including the RNA replicon model (Sethna *et al.*, 1989), looping out of full-length minus RNA strands into subgenomic minus strands, leader-primed transcription from subgenomic minus strands (Sawicki and Sawicki, 1990), and cotranscriptional splicing of independently transcribed leader RNA and mRNA body sequences (Spaan *et al.*, 1988). Currently, there is little genetic data to support any of the different models for MHV transcription.

In this article, we have initiated a genetic approach to characterize the location and function of the MHV polymerase genes and to examine the mechanism for MHV transcription. Mutants from the five RNA⁻ complementation groups are incapable of transcribing any detectable levels of viral RNA when infection is initiated at the restrictive temperature. However, temperature shift experiments after the onset of mRNA synthesis clearly revealed the presence of at least two classes of RNA⁻ mutants. Complementation groups A, B, D, and E were defective in functions required during mRNA and genome transcription indicating that at least four viral genes may participate directly or indirectly in positive-sensed transcription.

Previous studies suggest that the group D mutants synthesize the smallest of the four leader RNAs, but not mRNA after shift to restrictive temperature (Baric *et al.*, 1985). These data suggest that the group D cistron functions after leader transcription, but prior to mRNA synthesis. The group E mutants continue to synthesize low levels of negative-stranded RNA after shift, but not mRNA or genome. The exact function of this cistron in mRNA synthesis is unclear and will require additional study. The most likely explanation for the difference in steady state levels of minus strand RNA at permissive and restrictive temperature observed with the group E mutants is that progeny plus strands probably act as templates in successive rounds of negative strand RNA synthesis and that the concentrations of these mRNAs are greatly reduced following shift to restrictive temperature. Additional evidence that the group E cistron continues minus strand synthesis will require direct monitoring of the rates of negative strand synthesis and

analysis of replicative form RNAs. The function of the A and B cistrons is unclear, but genetic mapping studies suggest that the group A mutants map in the autoprotease activity located 3.9–5.1 kb from the 5' end of the genome (Baric *et al.*, 1990).

The group C cistron probably encodes an early function in MHV transcription which affects the overall rate of positive-sensed RNA synthesis. After shift to the restrictive temperature, group C mutants are probably defective in the ability to increase levels of negative-sensed RNA. It seems unlikely that the inability to increase the steady state levels of negative-stranded RNA after temperature shift is due to increased degradation since the group E mutants continue to synthesize low levels of negative-stranded RNA under identical treatment conditions. Rather, these results are consistent with the interpretation that the group C cistron encodes a protein selectively required in minus strand synthesis. At this time, we cannot discern whether the product of the group C cistron is temperature sensitive or whether the cleavage of the polyprotein encoding the group C cistron is defective at the restrictive temperature. Direct monitoring of the rates of negative strand synthesis and determining the amount of full-length and subgenomic replicative form RNAs after shift will provide additional proof that the group C cistron functions in minus strand synthesis and should provide genetic support for the presence of subgenomic RFs (Sethna *et al.*, 1989; Sawicki and Sawicki, 1990).

Previous kinetic studies of coronavirus minus strand synthesis have produced variable results. Brayton *et al.* (1982) detected an early minus strand polymerase activity at 1 but not at 6 hr postinfection in MHV-infected cells. At a high m.o.i. (50–100), Sawicki and Sawicki (1986) were unable to detect MHV negative-stranded RNA synthesis until 3 hr postinfection with the peak rate occurring at 6 hr postinfection. After this time, minus strand synthesis continued at 20% of the maximum rate. Peak quantities of the negative-stranded RNA occurred near the end of the infectious cycle. Sethna *et al.* (1989) found peak levels of TGEV minus strand RNA occurring at 4 hr postinfection. Under our conditions (m.o.i. 4), we were first able to detect minus strand RNA by 5 hr postinfection in DBT cells with peak levels occurring between 7 and 9 hr postinfection at 32° (Fig. 6D). In this study, the delayed appearance of negative strand RNA synthesis can probably be explained by growth in a different cell line, a lower m.o.i. and incubation temperature, and sensitivity of the probe. Since we monitored the steady state levels of minus strand RNA, a temporally distinct early minus strand polymerase should result in relatively constant levels of negative-stranded RNA between 5 and 11 hr postinfection. While we cannot rule out the

possibility of an early minus strand RNA polymerase activity in infected cells, our results are more consistent with the findings of Sawicki and Sawicki (1986) suggesting that peak levels of minus strand RNA occur near the end of the growth cycle. This interpretation is further supported by temperature shift experiments at different times postinfection which suggest that the negative strand defect in the group C cistron affects the overall rate of positive strand synthesis later in infection (Fig. 4).

These data support previous findings for the presence of a distinct polymerase activity involved in the synthesis of negative-stranded RNA (Brayton *et al.*, 1984; Sawicki and Sawicki, 1986). It is not surprising that a specific RNA polymerase activity may function during MHV negative strand synthesis. Previous studies with sindbis ts mutants indicate that two complementation groups function in negative strand synthesis (Sawicki *et al.*, 1981a,b). One cistron probably functions in the initiation of negative strand synthesis while the second cistron represents an elongation component of the viral replicase required in both positive and negative strand RNA synthesis (Sawicki *et al.*, 1981a,b; Hahn *et al.*, 1989a,b). At this time it is unclear whether the group C cistron of MHV encodes a distinct polymerase containing initiation and elongation activities or whether it encodes a product that interacts with other polymerase genes to form a negative strand RNA polymerase.

The location and orientation of all the MHV-A59 complementation groups used in this study is unclear, but T1 fingerprint analysis of recombinant viruses suggest that groups A, C, and D map in the polymerase gene at the 5' end of the genome (Keck *et al.*, 1987, 1988b; Lai *et al.*, 1985) and that the group F RNA⁺ mutants map in the S glycoprotein gene roughly 7–8 kb from the 3' end of the genome (Makino *et al.*, 1987, 1986a). Recently, a genetic recombination map of the MHV-A59 complementation groups has been established which indicates that five or more genetic functions are encoded in the polymerase region at the 5' end of the genome (Baric *et al.*, 1990).

ACKNOWLEDGMENTS

We thank Phyllis Driscoll for excellent technical assistance, Gillian Harris for manuscript preparation, and Jim Keck and Bob Johnston for helpful comments and criticisms. This investigation was supported by Public Health Service Grants AI 23946 and NS 18146, National Science Foundation Grant DMB-89-17148, and a grant from the American Heart Association (ANA 87-1135). This work was done during the tenure of an Established Investigator of the American Heart Association (AHA 89-0193) (R.S.B.).

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