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MHV nucleocapsid synthesis in the presence of cycloheximide and accumulation of negative strand MHV RNA

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Summary

We have found that genomic RNA synthesis is inhibited by cycloheximide in cells infected with mouse hepatitis virus, strain A59 (MHV-A59), in agreement with previously published results (Sawicki, S.G. and Sawicki, D.L. (1986) J. Virol. 57, 328–334). In the present study, the fate of the residual genomic RNA synthesized in the presence of cycloheximide was determined. Nearly all of the genomic RNA synthesized in the presence of drug was incorporated into nucleocapsid structures, suggesting that even in the absence of protein synthesis, genomic RNA synthesis and encapsidation are coupled in MHV-infected cells. Sufficient free nucleocapsid N protein was available for this purpose, since the pool of soluble N protein was determined to decay with a half-life of approximately one hour.

Negative strand RNA is the template for the synthesis of both genomic and subgenomic positive strand RNA, and would be predicted to accumulate primarily during the early phases of the lytic cycle. In agreement with this prediction, negative strand RNA accumulated during the first 5–6 h of infection, with little additional accumulation occurring over the next 2.5 h. In marked contrast, positive strand RNA increased 5–6-fold over the same 2.5 h period. These results, taken in conjunction with published data, suggest that negative strand RNA is synthesized during the early period of the infectious cycle and is stable in infected cells and also suggest that treatment with cycloheximide at late times does not inhibit positive

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strand RNA synthesis indirectly by blocking the formation of negative strand templates.

mouse hepatitis virus, coronavirus, viral replication

Introduction

Mouse hepatitis virus (MHV), a coronavirus, contains a polyadenylated, infectious RNA genome of approximate molecular weight 6×10^6 (Leibowitz et al., 1981; Siddell et al., 1983; Spaan et al., 1981). Intracellular viral RNA consists of a nested set of mRNAs with overlapping 3′ ends (Cheley et al., 1981; Lai et al., 1981; Leibowitz et al., 1981; Siddell et al., 1983; Spaan et al., 1982). The largest intracellular viral RNA corresponds in length to genomic RNA and is called RNA 1; the other six intracellular RNAs are labelled respectively from largest to smallest, RNA 2 to RNA 7. Each RNA has the same 72 base sequence at its 5′ end ("leader RNA"), derived from the 5′ end of the genome (Lai et al., 1983, 1984; Spaan et al., 1982, 1983).

After viral penetration and uncoating, the first step in replication is believed to be the translation of input RNA into an RNA-dependent RNA polymerase. This polymerase is then believed to catalyze the synthesis of a complementary copy of the input RNA (negative strand) which serves as template for the synthesis of the seven positive strand mRNAs and previrion RNA. Only full length negative strand RNA has been detected in infected cells (Lai et al., 1982) and presumably serves as the template for both mRNA and previrion RNA. Negative strand RNA is always contained in viral replicative intermediates and is synthesized at maximal rates at 5–6 h p.i. in tissue culture cells (Sawicki and Sawicki, 1986).

Maximal synthesis of genomic and subgenomic RNA in MHV-infected cells requires continuous protein synthesis at all times after infection (Brayton et al., 1982; Mahy et al., 1983; Sawicki and Sawicki, 1986). In this report, we show that the residual genomic RNA synthesized in the presence of cycloheximide is incorporated into nucleocapsids. We also show that negative strand RNA accumulates primarily during the first few hours of the lytic cycle, suggesting that the effect of cycloheximide on subgenomic and genomic RNA synthesis cannot be explained by the lack of negative strand templates.

Materials and Methods

Virus and cells

MHV-A59, a weakly hepatotropic strain adapted for tissue culture cells and used in all experiments was kindly provided by Dr. S. Weiss. The virus was plaque purified twice and grown in monolayer cultures of 17Cl-1 mouse fibroblast cells in Dulbecco's MEM (DMEM) supplemented with 2% fetal calf serum and 0.02 M

Hepes, pH 6.5, for 24–48 h. Viral stocks were prepared from the supernatant and titrated on 17Cl-1 or L-2 cell monolayer cultures.

Analysis of intracellular RNA

L-2 cells grown in 60-mm tissue culture dishes were infected at a m.o.i. of 10. After adsorption for 30 min at 37°C, DMEM supplemented with 2% fetal calf serum and 0.02 M Hepes, pH 6.5, was added to each dish. Actinomycin D (5 μg/ml), a gift from Merck, Sharpe and Dohme, was added to each plate at the same time. Cells were labelled with [5,6-^{3}H]uridine (48 Ci/mM, Amersham Corp.) at 100 μCi/ml as described in the figure captions. After labelling, cells were washed with cold phosphate-buffered saline (PBS), dislodged from the dishes with a rubber policeman and pelleted. The cells were lysed in NTM (0.1 M NaCl, 0.01 M Tris, pH 7.4, 0.001 M $MgCl_2$, 0.5% NP40) to which vanadyl sulfate-ribonucleoside complexes (10 mM final concentration) were added just prior to use (Berger and Birkemeier, 1979). We found that viral RNA, particularly RNA 1, was partially degraded if these complexes were omitted from the lysis buffer. Cytoplasm was prepared, sodium dodecyl sulfate (SDS) added to 0.5% and RNA extracted with phenol containing 0.1% 8-hydroxyquinoline and chloroform. RNA was precipitated with ethanol at −20°C.

RNA was analyzed by 1% agarose gel electrophoresis under denaturing conditions (6% formaldehyde) (Lehrach et al., 1977). RNA was denatured by treatment at 60°C for 5 min in a solution containing 6% formaldehyde, 50% formamide, 20 mM Mops, pH 7. After electrophoresis was completed, gels were treated with En^{3}Hance (New England Nuclear Corp.) and exposed to Kodak XAR film at −70°C, for the times indicated in the figure captions.

Preparation of [^{3}H]uridine-labelled nucleocapsids

Infected cells were labelled with [^{3}H]uridine (100 μCi/ml), washed with PBS, and resuspended in RSB (0.01 M NaCl, 0.01 M Tris, pH 7.6, 0.0015 M $MgCl_2$) supplemented with vanadyl sulfate-ribonucleoside complexes. Cells were lysed with 0.5% NP40 and cytoplasm prepared. EDTA was added to a final concentration of 0.01 M and each sample was layered on a 15–30% sucrose gradient containing RSB and 5 mM vanadyl sulfate-ribonucleoside complexes. Centrifugation was at 38000 rpm in the Beckman SW40 rotor at 4°C for the times indicated in the figure captions. Gradients were fractionated and assayed for TCA-precipitable radioactivity.

RNA dot and slot blot analyses

Unlabelled RNA was isolated from infected cells at the times indicated and prepared for either dot blot analysis as described (Thomas, 1980) or slot blot analysis as suggested by the manufacturer (Schleicher and Schuell). For the slot blot analysis, RNA was treated at 65°C for 5 min before application to nitrocellulose paper, in 4.6 M formaldehyde and 7.5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M Na citrate). The nitrocellulose was baked, prehybridized and hybridized as described (Thomas, 1980). Prehybridization and hybridization were performed at 42°C in 50%

formamide, 6 × SSC, 20 mM phosphate buffer, pH 6.0, 250 μg/ml denatured, sonicated calf thymus DNA, 0.1% SDS, 0.1% Ficoll, 0.1% polyvinylpyrrolidone and 0.1% bovine serum albumin.

The probe used in this analysis was a 500 bp sequence from the 3′-end of MHV-JHM. This portion of the genome is approximately 95% homologous between the A59 and JHM strains (Skinner and Siddell, 1984). Virion RNA was originally cloned into pBR322 by standard techniques (Maniatis et al., 1982) and then into M13 phage (Messing, 1983). Phage containing positive and negative strand sequences were selected by annealing with DNA complementary to virion RNA.

DNA was isolated by phenol extraction and labelled as described (Messing, 1983). Briefly, 120 ng template and 1.2 ng hybridization probe primer (17 base primer, P-L Biochemicals) were annealed together at 55°C for 10 min in H buffer (6 mM Tris, pH 7.5, 6 mM NaCl, 6 mM $MgCl_2$, 1 mM dithiothreitol) in 8 μl. After cooling, dGTP, dATP and TTP (final concentration of 50 μM), 20 μCi [^{32}P]dCTP (> 3000 Ci/mM, Amersham Corporation) and 5 U DNA polymerase (Klenow fragment, Boehringer-Mannheim Biochemicals) were added. The reaction was incubated at 15°C for 60 min and terminated with EDTA. The labelled DNA was used without further purification. Under these conditions of synthesis only the M13 DNA becomes double stranded and the insert portion remains single stranded. Final specific activity was $2–4 \times 10^8$ dpm/μg.

After annealing, the blots were washed as described (Thomas, 1980); the final wash was at 55°C in 0.2 × SSC, 10 mM phosphate buffer, 0.1% SDS and 1 mM EDTA. The blots were exposed to Kodak XAR at −70°C as described in the figure legends.

Analysis of proteins

For labelling of proteins, four 60-mm dishes of infected cells were washed with methionine-free DMEM supplemented with 2% dialyzed calf serum and resuspended in the same medium containing 100 μCi/ml [^{35}S]methionine for 10 min. One sample was immediately harvested, and unlabelled methionine (final concentration 1 mM) was added to the other three dishes. These samples were incubated for an additional 15, 30 or 60 min. For harvesting, cells were washed with ice-cold PBS three times and processed as described above for the preparation of nucleocapsids. In these experiments, centrifugation was at 45000 rpm at 4°C for 3 h in the Beckman SW56 rotor in order to pellet the nucleocapsids. Gradients were fractionated and 5 μl of each sample assayed for TCA-precipitable radioactivity. The soluble proteins, present in the top two fractions of the gradients, were concentrated with a Centricon microconcentrator (Amicon), and the nucleocapsid proteins, present in the pellet, were resuspended in 100 μl of NTE buffer (0.1 M NaCl, 0.01 M Tris, pH 7.4, 0.001 M EDTA) containing 0.5% SDS. Portions of each sample were analyzed by SDS-polyacrylamide gel electrophoresis as previously described (Laemmli, 1970; Maizel, 1971; Denison and Perlman, 1986).

Densitometry

Autoradiographs were scanned with a Shimadzu CS-930 dual-wavelength TLC scanner.

Results

Effect of cycloheximide on nucleocapsid synthesis

Maximal RNA synthesis in MHV-infected L-2 cells occurred at 5–8 h p.i., and the presence of cycloheximide inhibited the incorporation of [^{3}H]uridine into viral RNA at all times after infection, in agreement with previous reports (Brayton et al., 1982; Mahy et al., 1983; Sawicki and Sawicki, 1986). We also confirmed the results of Sawicki and Sawicki (1986) showing that the syntheses of both genomic and subgenomic RNA were inhibited by cycloheximide at all times after infection, although we found that the synthesis of genomic RNA was slightly more sensitive to protein synthesis inhibition than was that of the subgenomic RNAs. Thus in the experiment shown in Fig. 1B, the synthesis of RNA 1 (genomic RNA) and RNA 7 (the most abundant subgenomic RNA) was inhibited by 82% and 60%, respectively when measured by densitometry. Cycloheximide inhibited protein synthesis by approximately 97% at the concentration used in these experiments.

Intracellular genomic RNA is contained in viral nucleocapsid structures (Robb and Bond, 1979; Spaan et al., 1981) which are believed to be the precursors to infectious virus. To determine the effect of cycloheximide on the synthesis of these structures, cytoplasm was prepared from control and drug-treated infected L-2 cells at 5 h p.i. and divided into two parts. A portion of each sample was assayed for the presence of nucleocapsids by sucrose gradient sedimentation analysis. An EDTA-resistant structure sedimenting at about 200 S was isolated (Fig. 1A) and shown to contain only RNA 1 (Fig. 1B), in agreement with a previous report (Spaan et al., 1981). Pretreatment with cycloheximide caused approximately an 85% inhibition in the appearance of RNA 1 in the nucleocapsid structure. RNA was extracted from the remainder of each sample and analyzed directly by gel electrophoresis (Fig. 1B). Since the syntheses of RNA 1 and viral nucleocapsids were inhibited to nearly the same extent (82% and 85% respectively), this suggested that the residual genomic RNA produced in the presence of cycloheximide was incorporated into nucleocapsids.

To determine more directly if most of genomic RNA synthesized in treated cells was present in nucleocapsids, ribonucleoprotein particles were fractionated on sucrose gradients and each individual fraction was assayed for the presence of RNA 1. As shown in Fig. 2, RNA 1 could only be detected in nucleocapsids and not in any slower sedimenting particles. This result indicated that the synthesis of progeny RNA and its incorporation into nucleocapsid structures remained coupled in the presence of cycloheximide.

Kinetics of association of N protein with soluble and nucleocapsid pools

These results suggested that a substantial pool of free nucleocapsid N protein existed in infected cells. To measure the size of this pool, cells were labelled for 10 min with [^{35}S]methionine and chased for various times with a vast excess of unlabelled methionine. No increase in total radioactivity occurred after the initial pulse period, proving that the chase was effective. Samples containing soluble and nucleocapsid proteins were prepared and analyzed by polyacrylamide gel elec-

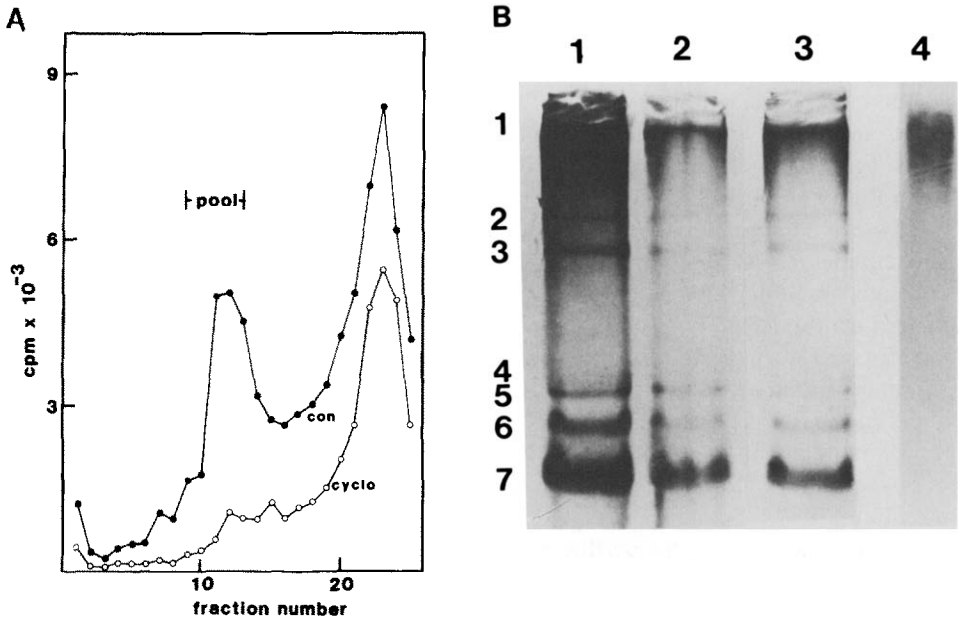

Fig. 1. Effect of cycloheximide on nucleocapsid synthesis. (A) Cytoplasm was prepared from control and cycloheximide-treated L-2 cells labelled with [^{3}H]uridine (100 μCi/ml) at 5 h p.i. for 90 min. Each sample was split and one portion analyzed by sucrose gradient centrifugation for 75 min at 39000 rpm in the Beckman SW 40 rotor as described in Materials and Methods. The gradients were fractionated into 0.5 ml fractions. 0.05 ml was removed from each fraction and assayed for TCA-precipitable radioactivity. The top of the gradient is at the right. ●———●, control; ○———○, cycloheximide added 10 min prior to labelling. (B) RNA was extracted from the second part of each sample with phenol/chloroform and analyzed by gel electrophoresis as described in Materials and Methods (lane 1, control; lane 2, cycloheximide-treated, 21 h exposure; lane 3, lane 1 exposed for 9 h). Lane 4: the nucleocapsid structure from the control sample indicated in Fig. 1A ("pool") was deproteinized with phenol/chloroform and analyzed by agarose gel electrophoresis. This gel was exposed for 72 h. The seven intracellular viral RNAs are labelled on the gel.

trophoresis as described in Materials and Methods (Fig. 3). The three major structural proteins, N, E1 and E2, with molecular weights 60000, 23000 and 180000, respectively, were evident in the soluble fraction after the 10 min label, and were absent from the lysates from mock-infected cells. These proteins could also be selectively precipitated with specific antiserum directed against MHV-A59 viral proteins (data not shown).

After short periods of labelling with radioactive amino acids, the intracellular pool of N protein in MHV-infected cells has been shown not to decrease during a subsequent 90–180 min chase with a large excess of unlabelled amino acids (Anderson et al., 1979; Siddell et al., 1981; Sturman and Holmes, 1983). In agreement with this observation, we found that approximately the same total amount of N protein was present at each time point, simplifying analysis. After the 10 min pulse, 95% of the N protein was present in the soluble fraction and 5% was

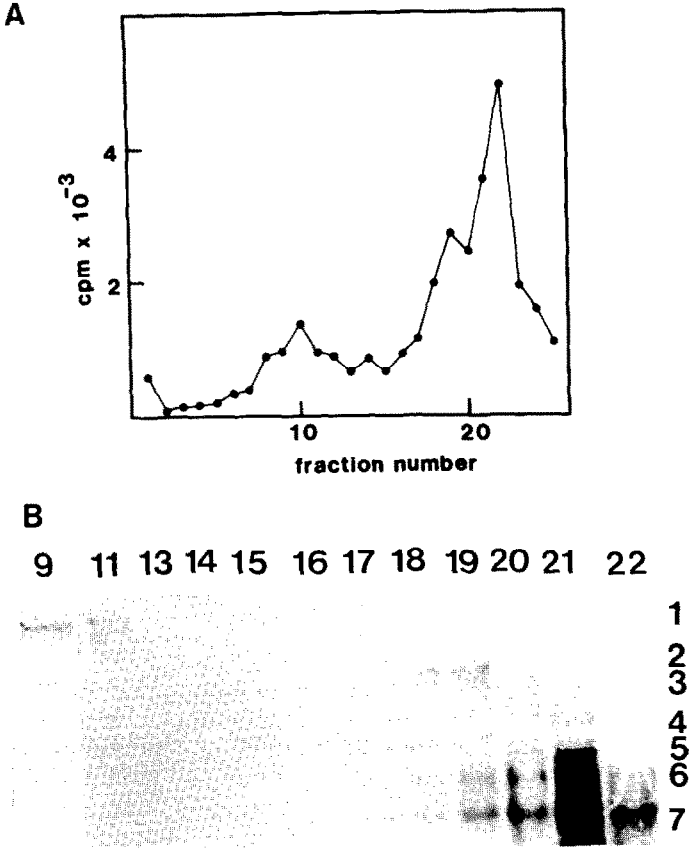

Fig. 2. Identification of structures in infected cells containing RNA 1 in the presence of cycloheximide. Cytoplasm was prepared from infected cells labelled with [^{3}H]uridine (100 μCi/ml) at 5.5 h p.i. for 60 min. Cycloheximide was added 10 min prior to labelling. Centrifugation was for 90 min at 39000 rpm in the Beckman SW 40 rotor. The gradients were fractionated and 0.05 ml assayed for TCA-precipitable radioactivity (top panel, top of the gradient is at the right). Each indicated fraction was deproteinized with phenol/chloroform and analyzed by gel electrophoresis (bottom panel). The seven intracellular viral RNAs are marked.

in the nucleocapsid fraction (Fig. 4). After a 60 min chase, the N protein was present in nearly equal amounts in the soluble and nucleocapsid fractions (Fig. 4). This experiment showed that a sufficient pool of free N protein was present in infected cells to support the continued incorporation of newly synthesized RNA into nucleocapsids even in the absence of de novo protein synthesis, especially since genomic RNA synthesis was reduced under these conditions.

Accumulation of negative strand RNA

It is possible that the observed inhibition of positive strand RNA synthesis by cycloheximide might be partially due to an inhibition of the synthesis of negative

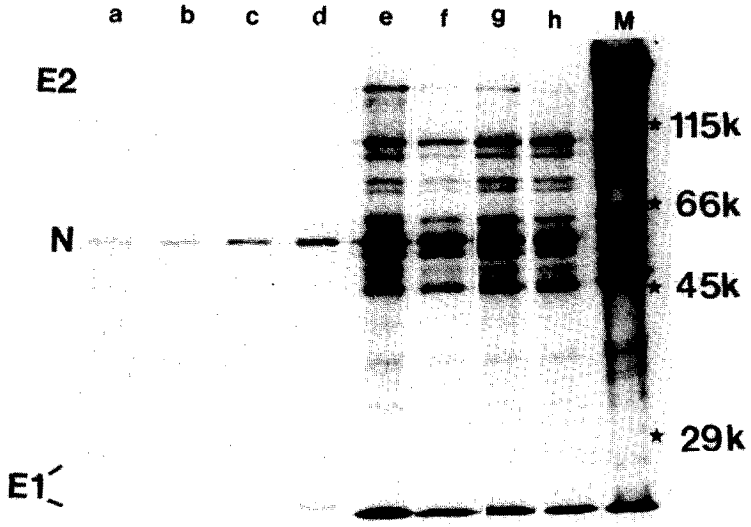

Fig. 3. Presence of N protein in soluble and nucleocapsid cell fractions. Cytoplasm was prepared from infected cells labelled with [^{35}S]methionine at 5.5 h p.i. for 10 min and chased with unlabelled methionine for 0, 15, 30 or 60 min. Nucleocapsids were prepared as described in Materials and Methods, and the soluble and nucleocapsid proteins analyzed by SDS-PAGE using 10% polyacrylamide gels. Lanes a–d and e–h show nucleocapsid and soluble proteins respectively. Lanes a, e are pulse samples, and chase samples are shown in b, f (15 min), c, g (30 min) and d, h (60 min). Lane M shows a sample from mock-infected cells labelled for 60 min with [^{35}S]methionine. Molecular weights of unlabelled markers run in parallel and stained with Coomassie blue are also indicated, as are the major viral proteins. This gel was exposed for 17 h.

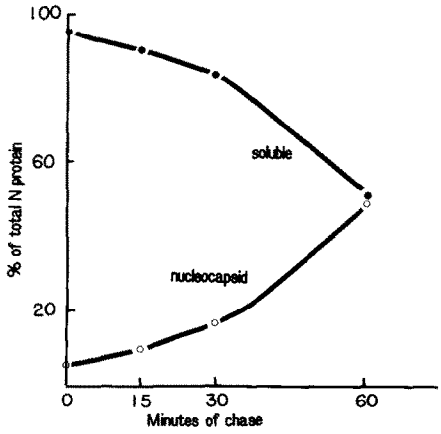

Fig. 4. Time course of appearance of N protein in soluble and nucleocapsid cell fractions. The gels shown in Fig. 3 were scanned with a densitometer, and the fraction of N protein in the nucleocapsid and soluble fractions determined. ○——○, nucleocapsid proteins; ●——●, soluble proteins.

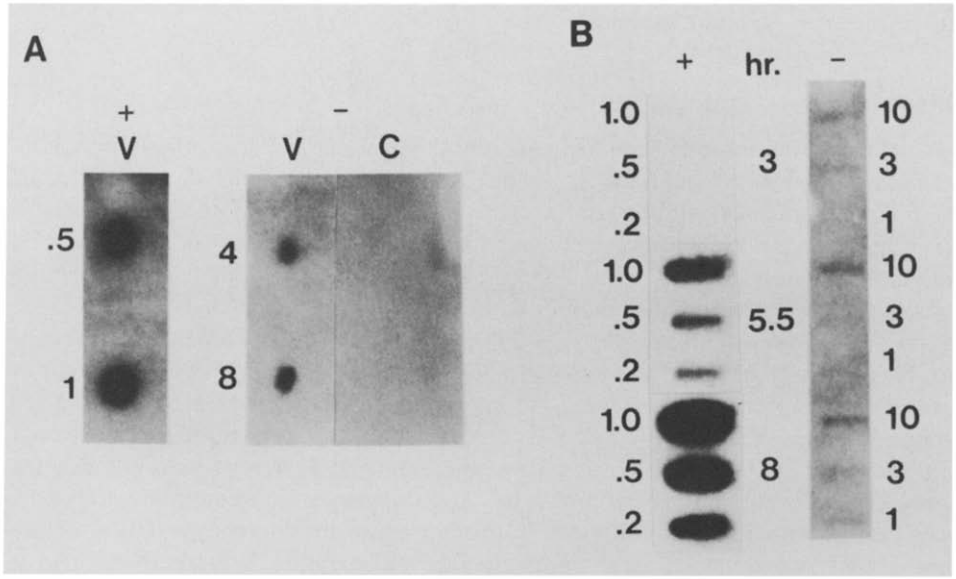

Fig. 5. Slot blot analysis of infected cell RNA. (A) RNA was prepared from uninfected and infected cells (5 h p.i.) and analyzed by dot blot analysis as described in Materials and Methods. 0.5 and 1.0 μg of RNA were assayed for positive strand sequences whereas 4.0 and 8.0 μg were assayed for negative strand sequences. The blots were exposed for 3 days. v, virus-infected RNA; c, control RNA. (B) RNA was prepared from infected cells at 3, 5.5 and 8 h p.i. and analyzed by slot blot analysis as described in Materials and Methods. 0.2, 0.5 and 1.0 μg of RNA were assayed for positive strand sequences at each time point, whereas 1.0, 3.0 and 10.0 μg were assayed for negative strand RNA. The left and right blots were exposed for 1 day and 7 days, respectively.

strand templates. Thus, in the next set of experiments, we measured the amount of positive and negative strand RNA present in infected cells prior to (3 h p.i.), at the beginning of (5.5 h p.i.), and at the end of (8 h p.i.) maximal viral RNA synthesis. For this purpose, cloned M13 DNA probes specific for positive and negative strand RNA sequences were prepared as described in Materials and Methods.

These probes were annealed to unlabelled RNA isolated from uninfected and infected cells and fixed to nitrocellulose filters (Fig. 5). No annealing occurred with RNA from uninfected cells (Fig. 5A). As expected, positive strand RNA accumulated throughout the infection and increased approximately 5–6-fold between 5.5 h and 8 h p.i. (Fig. 5B).

On the other hand, negative strand RNA was present in very low quantities throughout the infection although it could be detected as early as 3 h p.i. At 5.5 h p.i., negative strand RNA was clearly present with little additional accumulation (less than 2-fold) occurring by 8 h p.i. From this and other experiments, we estimated that negative strand RNA represented approximately 1% of RNA at 5 h p.i. and less than 0.5% at 8 h p.i. We concluded that the inhibition of positive strand

synthesis by cycloheximide at late times after infection was most likely not due to a lack of negative strand templates.

Discussion

Cycloheximide inhibits both genomic and subgenomic RNA synthesis in MHV-infected cells (Sawicki and Sawicki, 1986; Fig. 1). The genomic RNA synthesized in the presence of cycloheximide continues to be incorporated into nucleocapsids, and this suggests that the synthesis of genomic RNA and its encapsidation may be coupled. In cells infected with vesicular stomatitis virus (VSV), cycloheximide causes a rapid cessation of genomic RNA synthesis (Perlman and Huang, 1973; Wertz and Levine, 1973) and the rate-limiting protein appears to be the nucleocapsid N protein (Patton et al., 1984). In VSV-infected cells, 50% of N protein is present in the soluble pool after a 2–10 min label and this decreases to 8–25% after 30 min of chase (Knipe et al., 1977; Hsu et al., 1979). In contrast, in MHV-infected cells, the pool of free N protein is much larger, since 95% of N protein was present in the soluble fraction after a 10 min label, and this decreased to approximately 50% over the next hour. Thus, sufficient N protein exists for the encapsidation of the genomic RNA synthesized in the presence of cycloheximide. Whether N protein is rate-limiting for genomic RNA synthesis in MHV-infected cells remains to be determined.

Cycloheximide also inhibits subgenomic RNA synthesis in MHV-infected cells, in contrast to its effect on transcription in cells infected with other viruses such as VSV or Semliki Forest virus, in which mRNA synthesis does not decline, but rather remains constant in the absence of protein synthesis (Perlman and Huang, 1973; Wertz and Levine, 1973; Sawicki and Sawicki, 1980). The synthesis of MHV subgenomic RNA is believed to include synthesis of a leader sequence, which in turn serves as primer for the body of each RNA (Spaan et al., 1983; Lai et al., 1984). The effect of cycloheximide on subgenomic RNA synthesis may reflect lability either of the viral polymerase or of some protein involved in the synthesis or processing of leader RNA. Similarly, the effect of this drug on genomic RNA synthesis may reflect a lability in one of these proteins rather than a limited supply of N protein.

Several aspects of negative strand MHV RNA synthesis have been elucidated. First, only genome-sized RNA of this polarity is found in infected cells (Lai et al., 1982). Second, negative strand RNA is synthesized maximally at 5–6 h p.i. (Sawicki and Sawicki, 1986), and is the chief product observed when viral polymerase activity is assayed at 1 h p.i. in vitro (Brayton et al., 1984). Third, our results show that negative strand RNA accumulates primarily during the early stages of the infectious cycle. These results together suggest that negative strand RNA is synthesized at early times during the infectious cycle and is a stable species of RNA in infected cells. The results also suggest that cycloheximide does not inhibit positive strand RNA synthesis indirectly via inhibition of negative strand synthesis, since the production of positive strand RNA remains sensitive to cycloheximide at times when the synthesis and accumulation of negative strand RNA is minimal.

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