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Intracellular Murine Hepatitis Virus-Specific RNAs Contain Common Sequences

STEVE CHELEY, ROBERT ANDERSON, MARGARET J. CUPPLES, EDWIN C. M. LEE CHAN, AND VINCENT L. MORRIS[1]

Department of Microbiology and Immunology, University of Western Ontario, London, Ontario N6A 5C1, Canada

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A major polyadenylated viral RNA of approximately 0.8×10^6 daltons was isolated from murine hepatitis virus (A59)-infected cells by preparative polyacrylamide gel electrophoresis in formamide. This RNA was shown to encode the viral nucleocapsid protein by direct *in vitro* translation in a cell-free, reticulocyte-derived system. Single stranded ^{32}P-labeled complementary DNA was prepared from this RNA and was demonstrated to be virus specific. Using this complementary DNA in a Northern blotting procedure, we were able to identify six major virus-specific intracellular RNA species with estimated molecular weights of 0.8, 1.1, 1.4, 1.6, 3, and 4×10^6 daltons. All of these RNA species were polyadenylated. Our results support the idea that coronavirus-infected cells contain multiple intracellular polyadenylated RNAs which share common sequences.

INTRODUCTION

Coronaviruses have a single-stranded, polyadenylated, nonsegmented RNA genome which is infectious (Lai and Stohlman, 1978; Lomniczi, 1977; Lomniczi and Kennedy, 1977; Schochetman *et al.*, 1977; Wege *et al.*, 1978). Molecular weight estimates of this RNA range from 3 to 9×10^6 daltons (Lai and Stohlman, 1978; Lomniczi and Kennedy, 1977; MacNaughton and Madge, 1977; Tannock, 1973; Watkins *et al.*, 1975; Wege *et al.*, 1978). Although little is known about the RNA replication strategy of coronaviruses, Stern and Kennedy (1980a, b) have recently identified a set of five polyadenylated subgenomic RNAs in cells infected with the avian coronavirus, infectious bronchitis virus (IBV). Oligonucleotide fingerprint analysis of these RNAs suggested common sequences among them (Stern and Kennedy, 1980a, b). Evidence for multiple intracellular messenger RNAs (mRNAs) has also been presented in the case of another coronavirus, murine hepatitis virus (MHV). Siddell *et al.* (1980) reported the separation of polyadenylated RNAs from MHV (JHM strain)-infected cells by means of sucrose gradients; fractions from these gradients were able to direct the *in vitro* translation of two viral proteins. However, the limited degree of resolution obtainable on sucrose gradients makes a more definitive analysis of MHV subgenomic RNAs highly desirable.

In the present report we provide firm evidence for the presence, in MHV-infected cells, of a set of six overlapping, polyadenylated viral RNAs. Our results provide independent support for the "nested set" concept of coronavirus mRNAs, as advanced by Stern and Kennedy (1980a, b).

MATERIALS AND METHODS

Cells and virus. The A59 strain of MHV was obtained from the American Type Culture Collection. Virus was propagated and plaqued on the L-2 strain (Rothfels *et al.*, 1959) of mouse fibroblasts.

[1] To whom reprint requests should be addressed.

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Measurement of [^{3}H]uridine incorporation into infected cells. Confluent cultures of L-2 cells in 35-mm petri dishes were inoculated at a multiplicity of infection (m.o.i.) of 5 and incubated at 37° in minimum essential medium (MEM) supplemented with 5% fetal calf serum (FCS) and containing actinomycin D (1 μg/ml). At intervals, medium was replaced with 0.5 ml of MEM containing actinomycin D and [5-^{3}H]uridine (15 μCi/ml). After 30 min incubation at 37° the cell monolayers were washed with phosphate-buffered saline (PBS) and the cells lysed with 0.5% sodium dodecyl sulfate (SDS). After the addition of 10% trichloroacetic acid to cell lysates, RNA was trapped by filtration through Whatman 3MM filter disks and the radioactivity determined by scintillation spectrometry.

Isolation of ^{32}P-labeled infected cell RNA for formamide gel electrophoresis. For large-scale preparation of infected cell RNA, seventy-five 100-mm petri dishes of confluent L-2 cells were inoculated at a m.o.i. of approximately 2 and subsequently incubated at 37° in MEM supplemented with 5% FCS and containing actinomycin D (1 μg/ml). At 3.5 hr postinoculation (PI) the medium in five petri dishes was replaced with phosphate-free medium containing actinomycin D and [^{32}P]phosphate (800 μCi/ml). At 8.5 hr PI, at which time the infected cell monolayers were completely fused, medium was removed from all 75 dishes and the cells washed once with PBS. Cells were scraped with a rubber policeman in a total of 36 ml RSB (10 m*M* Tris, 10 m*M* NaCl, 1.5 m*M* $MgCl_2$, pH 7.4) and extracted with chloroform/phenol/isoamyl alcohol according to the procedure of Rose and Knipe (1975). RNA was recovered from the aqueous phase by the addition of 2 vol of ethanol and centrifugation for 20 min at 10,000 *g*. Prior to formamide gel electrophoresis RNA was poly(A) selected by passage through oligo(dT)-cellulose (see below).

Isolation of RNA for hybridization and transfer procedures. Cellular RNA was prepared according to the procedure of Strohman *et al.* (1977) as modified by Dr. G. Mackie (Department of Biochemistry, University of Western Ontario). Briefly, cell monolayers were washed with PBS, scraped into a solution of 7.6 *M* guanidine-HCl, 0.1 *M* potassium acetate, pH 7.5, and homogenized with a glass dounce in a bath of salt-ice. After addition of 0.6 vol of 95% ethanol and cooling for several hours at −20°, the mixture was spun at 3000 *g* for 10 min. The pellet was taken up in buffer containing 7.6 *M* guanidine-HCl, 0.02 *M* EDTA, and gently vortexed at room temperature. After addition of 2 *M* potassium acetate, pH 5, to a final concentration of 0.1 *M*, ethanol (0.5 vol) was added and the mixture centrifuged at 3000 *g* for 10 min. The pellet was dissolved in 20 m*M* EDTA, pH 7, and extracted at room temperature with 3 vol of chloroform/butanol (4:1, v/v). The aqueous phase was combined with two additional aqueous washings of the organic phase and made 3 *M* in sodium acetate, pH 6. After cooling at −20°, the mixture was centrifuged at 8700 *g* for 60 min to yield an RNA pellet which was subsequently precipitated twice from ethanol.

Oligo(dT)-cellulose chromatography. Ethanol-precipitated RNA was dissolved in application buffer (10 m*M* Tris, 0.5 *M* KCl, pH 7.5) and applied to a column of oligo(dT)-cellulose (0.5 g, Collaborative Research, Inc.). Stepwise elution with decreasing concentration of KCl was performed according to Aviv and Leder (1972). Fractions containing "unbound" (eluted with 0.5 *M* KCl buffer) and "bound" (eluted with KCl-free buffer) RNA were diluted with 2 vol of ethanol. Precipitated RNA was recovered by centrifugation for 20 min at 10,000 *g*.

Formamide-polyacrylamide gel electrophoresis. RNA samples dissolved in formamide-glycerol sample buffer (Duesberg and Vogt, 1973) were carefully applied to formamide-filled wells of a slab gel containing 3.5% acrylamide in formamide (Duesberg and Vogt, 1973). Electrophoresis was performed for 20 hr at 100 V. Gels were enclosed in Saran Wrap, frozen to facilitate subsequent manipulation, and autoradiographed for several hours at −70°.

Recovery of ^{32}P-labeled RNA from formamide gels. Using an autoradiogram as

template, ^{32}P-containing RNA bands were excised from the gel, macerated through an 18-gauge syringe needle, and eluted with extraction buffer (10 m*M* Tris, 0.4 *M* sodium acetate, 0.02% SDS, pH 7.4; Rose and Knipe, 1975). Eluted RNA was freed from minute gel debris by centrifugation at 10,000 *g* for 10 min, precipitated by addition of 2 vol of ethanol, and recovered by centrifugation for 3 hr at 100,000 *g*. Recovered RNA was re-poly(A) selected by oligo(dT)-cellulose chromatography as described above.

Preparation of complementary DNA. Complementary DNA (cDNA) was synthesized, using the procedure of Shank *et al.* (1978), in the presence of actinomycin D, calf thymus DNA primers, purified AMV polymerase (supplied by Dr. J. Beard and the Office of Program Resources and Logistics, National Cancer Institute) and [^{32}P]dCTP (Amersham, 400 Ci/mmol). The cDNA product was isolated according to the procedure of Cohen *et al.* (1979). Before use the cDNA was annealed (0.6 *M* NaCl, 68°) with 500 μg of uninfected L cell RNA to a C_rt of 230. The annealing mixture was then made 0.12 *M* in phosphate buffer and the single-stranded sequences were eluted from a hydroxyapatite column at 60° with 0.12 *M* phosphate buffer; the double-stranded sequences were then eluted from the column at 60° with 0.4 *M* phosphate buffer (Morris *et al.*, 1977). The fractions containing the single-stranded [^{32}P]cDNA (approximately 80% of the total radioactivity applied) were collected, pooled, and used for the subsequent experiments. Complementary DNA made with this technique has been shown to be representative of most, if not all, of a viral RNA by its ability to detect all the restriction endonuclease fragments of viral DNA with an efficiency equal to that of iodinated viral RNA (Ringold *et al.*, 1978).

Hybridization kinetic experiments. Hybridization was performed using the procedure of Varmus *et al.*, (1973). Hybridization mixtures contained 1150 cpm [^{32}P]cDNA per 15-μl aliquot, excess unlabeled RNA, and 0.6 *M* NaCl, 2 m*M* EDTA, 40 m*M* Tris-HCl, pH 7.4. Reactions were incubated at 68° under mineral oil in plastic tubes. Aliquots were removed and the percentage of single-stranded cDNA was determined using S1 nuclease (Calbiochem), which specifically digests single-stranded DNA, using the conditions of Leong *et al.* (1972).

Analysis using gel electrophoresis and RNA transfer procedures. RNA was denatured with glyoxal, subjected to electrophoresis in 1.5% agarose gels, transferred to diazobenzyloxymethyl (DBM) paper, and hybridized with [^{32}P]cDNA using a modification of the technique of McMaster and Carmichael (1977) and Alwine *et al.* (1977) as described in Coulter-Mackie *et al.* (1980). The [^{32}P]cDNA used in these experiments was preannealed (0.6 *M* NaCl, 68°) with 500 μg of uninfected L cell RNA to a C_rt of 150. The A59 virus-specific RNA species were visualized by autoradiography (Swanstrom and Shank, 1978).

DNA extraction and hybridization. Uninfected L cells were resuspended in 0.02 *M* Tris-HCl (pH 7.2), 0.01 *M* EDTA (pH 7). After incubating this suspension with Pronase (1 mg/ml, self-digested for 2 hr at 37°) and 1% SDS overnight, the aqueous phase was extracted three times with phenol-chloroform (1:1). The DNA was then dialyzed extensively against 0.05 *M* Tris-HCL (pH 7.2), 0.1 m*M* EDTA (pH 7). The solution was adjusted to 0.3 *M* NaOH, heated to 100° for 20 min, and quick-cooled in ice. After neutralization, the DNA was precipitated with ethanol at −20°, centrifuged at 16,300 *g*, and resuspended in 3 m*M* EDTA (pH 7). The DNA was then annealed with [^{32}P]cDNA as described by Morris *et al.* (1977) except cell DNA was 6.1 mg/ml in the annealing mix instead of 3.5 mg/ml. Single-stranded DNA was assayed with S1 nuclease as previously described (Leong *et al.*, 1972).

Cell-free protein synthesis. RNA was preparatively isolated from formamide-polyacrylamide gels as described above. Preparation of micrococcal nuclease-pretreated rabbit reticulocyte lysates and conditions for cell-free protein synthesis were essentially as described by Pelham and Jackson (1976). Following incubation, samples were prepared for electrophoresis on 10% polyacrylamide-SDS gels

(Laemmli, 1970) by treatment of the incubation mixtures (25 μl) with RNase A (final concentration, 4 μg/ml) for 15 min at room temperature followed by the addition of 25 μl dissociation buffer (7 m*M* Tris-phosphate, pH 6.8, 3.46 *M* mercaptoethanol, 30% glycerol, 6% SDS, and 0.006% bromphenol blue). Gels were dried and fluorographed according to the procedure of Bonner and Laskey (1974).

RESULTS

Viral RNA Synthesis in Actinomycin D-Treated Infected Cells

The inhibition by actinomycin D of host cell DNA-dependent RNA synthesis has been reported to be noninhibitory for the replication of coronaviruses (Tyrrell *et al.*, 1975). In order to confirm that viral RNA could be detected in actinomycin D-treated, A59-infected L cells, the incorporation of [^{3}H]uridine into acid-precipitable material was measured as a function of time following virus inoculation. The results, presented in Fig. 1, demonstrated a sharp increase in RNA synthesis between 5 and 6 hr PI. During this time, virus-induced cell fusion was evident in approximately 20% (5 hr PI) to 80% (6 hr PI) of the total cell monolayer. The subsequent decline in RNA synthesis observed at 7 hr PI was likely attributable to loss of cells from the infected monolayer due to the observed detachment of cell syncytia from the plastic substrate.

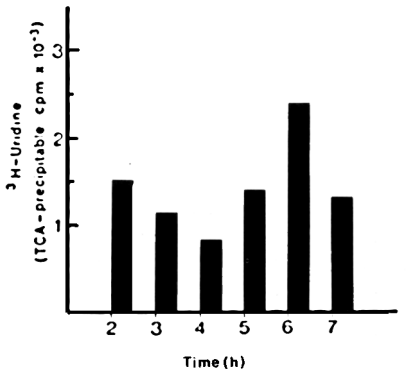

FIG. 1. Time course of viral RNA synthesis. Cultures of A59-infected L-2 cells were labeled with [^{3}H]uridine for 30-min periods in the presence of actinomycin D. Acid-precipitable radioactivity was determined as under Material and Methods.

Isolation of ^{32}P-Labeled Viral 0.8×10^6 Dalton RNA

RNA extracted from ^{32}P-labeled, actinomycin D-treated, infected cells was poly(A) selected and subjected to polyacrylamide gel electrophoresis in formamide. Autoradiography of the gel showed the presence of several labeled RNA bands, the smallest and also most heavily labeled of which had an apparent molecular weight of 0.8×10^6. The estimated molecular weight of this species was determined by assigning a molecular weight of 1.75×10^6 to 28 S mouse ribosomal RNA and 0.68×10^6 to 18 S mouse ribosomal RNA (McMaster and Carmichael, 1977). The 0.8×10^6 dalton RNA was preparatively purified by excision and extraction of the gel, followed by re-poly(A) selection. An aliquot of the purified 0.8×10^6 dalton RNA was examined for purity and intactness by reelectrophoresis. As can be seen in Fig. 2, the purified RNA migrates as a single spot with only negligible contaminants.

Identification of the 0.8×10^6 Dalton RNA as Viral Messenger RNA for the Nucleocapsid Protein

Translation of the purified 0.8×10^6 dalton RNA in an *in vitro*, reticulocyte-derived translation system yielded a single viral polypeptide of 53K daltons (Fig. 3). This polypeptide virtually comigrated with authentic viral nucleocapsid N protein present in an extract from [^{35}S]methionine-labeled A59-infected L cells. Identity between the *in vitro*-synthesized 53K protein and the authentic viral N protein was confirmed by peptide mapping (data not shown). In addition, a polypeptide of approximately 42K daltons was consistently detected in the *in vitro* translation extract, whether RNA was added or not (Fig. 3). This polypeptide is, therefore, likely to be the mRNA-independent 42K species orig-

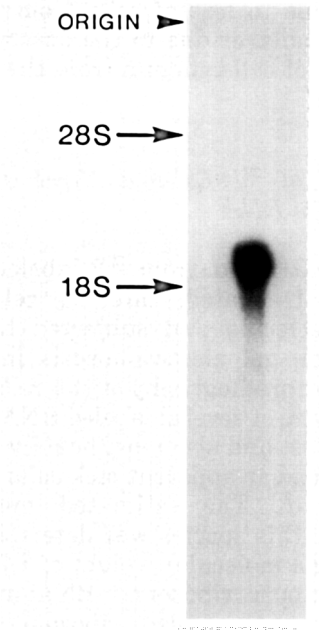

FIG. 2. Purity of the 0.8×10^6 dalton RNA. Total poly(A)-selected RNA from ^{32}P-labeled, A59-infected cells was separated by preparative gel electrophoresis and the 0.8×10^6 dalton RNA band extracted from the gel (see Materials and Methods). An aliquot of this purified RNA was reexamined by gel electrophoresis; an autoradiogram is shown.

inally described by Pelham and Jackson (1976) and which has been observed by numerous other investigators using the reticulocyte-derived translation system.

Characterization of cDNA Using the RNA Transfer Technique

We prepared single-stranded [^{32}P]cDNA against isolated 0.8×10^6 dalton RNA. This cDNA was then used, in an RNA transfer procedure, to identify intracellular A59-specific RNA. As expected, this cDNA annealed predominantly with a 0.8×10^6 dalton RNA species in A59-infected L cell RNA (Fig. 4, lane A). However, the cDNA also showed significant annealing with five other RNA species with estimated molecular weights of 1.1, 1.4, 1.6, 3 and 4×10^6. (Fig. 4, lane A). Similar results were obtained using poly(A)-selected A59-infected L cell RNA (Fig. 4, lane B), indicating that all the observed RNA species, which share homology with the [^{32}P]cDNA, were polyadenylated. These RNA species were visible in autoradiograms within several hours of exposure. In contrast to these results with infected L cell RNA, the cDNA showed no annealing with either uninfected L cell RNA (Fig. 4, lane C) or poly (A)-selected, uninfected L cell RNA (Fig. 4, lane D). Furthermore, the cDNA showed no annealing with RNA which was poly(A) selected from as much as one mil-

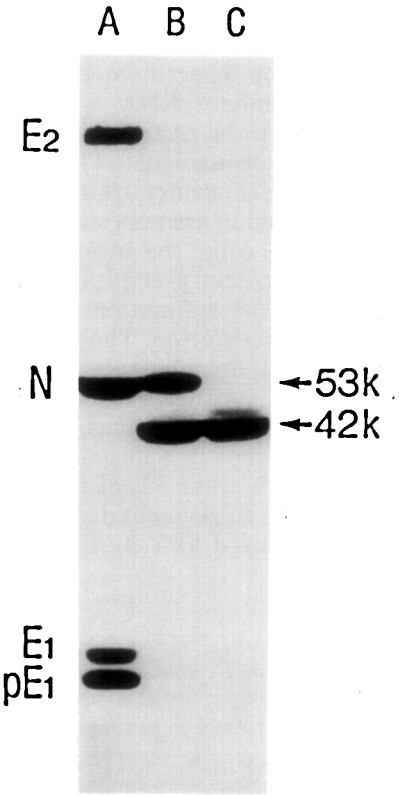

FIG. 3. Autoradiogram of an SDS-gel of [^{35}S]-methionine-labeled cell-free translation products from the 0.8×10^6 dalton RNA. Complete reticulocyte-derived translation mixtures were incubated in the presence (lane B) or absence (lane C) of 0.8×10^6 dalton RNA. For comparison, an extract from A59-infected L cells labeled *in vivo* for 30 min with [^{35}S]methionine is shown in lane A. Nomenclature of viral proteins is as proposed by Sturman *et al.* (1980) except for PE_1 which we have shown (Cheley and Anderson, in press) to be a precursor polypeptide to E_1.

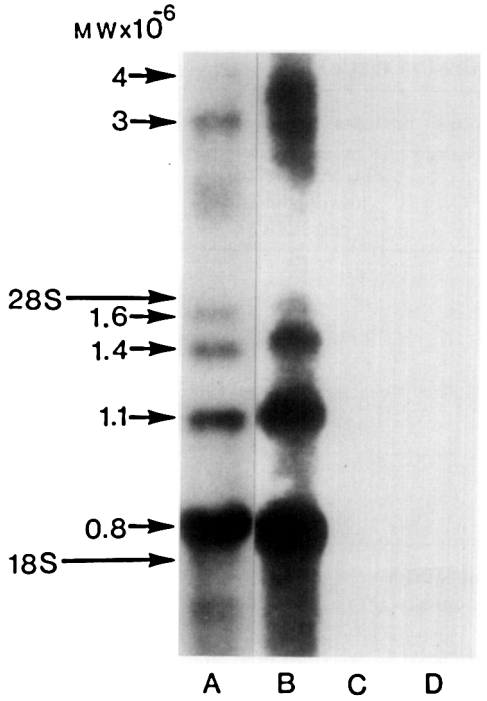

FIG. 4. Autoradiogram of A59-infected and uninfected L cell RNA species. A59-infected and uninfected L cell RNA was denatured with glyoxal, separated by electrophoresis in 1.5% agarose gels, transferred to diazobenzyloxymethyl paper and hybridized with A59-specific [^{32}P]cDNA. The A59 homologous RNA bands were visualized by autoradiography (see Methods). Lane A contained 30 μg of A59-infected L cell RNA; lane B contained 0.5 μg of poly(A)-selected, A59-infected L cell RNA (selected from 50 μg of RNA); lane C contained 30 μg of uninfected L cell RNA; lane D contained 0.5 μg of poly(A)-selected, uninfected L cell RNA (selected from 50 μg of RNA).

ligram of uninfected L cell RNA (data not shown).

Characterization of cDNA Using Hybridization Kinetics

The specificity of the cDNA prepared from 0.8×10^6 dalton RNA was further tested by annealing it with tRNA, uninfected L cell RNA, poly(A)-selected, uninfected L cell RNA, and A59-infected L cell RNA. Actinomycin D was added to the A59-infected L cells to prevent the expression of endogenous L cell genes (including retrovirus genes). The results are shown in Table 1. The tRNA hybridization data indicated that the [^{32}P]cDNA showed no significant self annealing. In addition, the [^{32}P]cDNA showed less than 10% annealing with either uninfected, or poly(A)-selected, uninfected L cell RNA. Thus, in agreement with the RNA transfer results, the cDNA had little if any homology with uninfected L cell RNA. In contrast, the cDNA annealed completely with A59-infected L cell RNA with a $C_rt_{1/2}$ of 5×10^{-2} (using data from Table 1). This result, using the calculation of Varmus *et al.* (1973), indicated that greater than 1000 genome equivalents were present per infected cell and was consistent with the observed rapid appearance of the major virus-specific RNA bands in the RNA transfer procedures (Fig. 4).

All A59-infected cell RNA was prepared from cells infected in the presence of actinomycin D to inhibit expression of host cell genes. In addition, an aliquot of the [^{32}P]cDNA was annealed with uninfected L cell DNA. This annealing was performed under conditions (0.6 *M* NaCl, 68°, $C_0t = 1.6 \times 10^4$, DNA/cDNA ratio = 9×10^7) that would allow single copy DNA to anneal to within 90% or more of completion (Morris *et al.*, 1977). However, no annealing above background was observed between the [^{32}P]cDNA and the L cell DNA. It is thus clear from the RNA transfer and the hybridization procedures that the major RNA bands identified by the [^{32}P]cDNA are indeed virus specific.

DISCUSSION

The results of the present study provide definitive proof for the presence of six major polyadenylated, viral RNAs in MHV-infected cells. The smallest of these RNAs, a species of 0.8×10^6 daltons, encodes the viral nucleocapsid protein. Moreover, and most significantly, this RNA contains sequences which are also contained within the five larger RNA species. Our results are thus analogous with the findings of Stern and Kennedy (1980a, b) that cells infected with the avian coronavirus, IBV, also contain a "nested set" of

TABLE 1

HYBRIDIZATION OF [^{32}P]cDNA AND UNLABELED RNA

	Percentage cpm resistant to S_1 nuclease			
C_rt[a]	A59-infected L cell RNA[b]	Uninfected L cell RNA	Poly(A)-selected uninfected L cell RNA	tRNA
0	7	4	—	—
0.01	27	—	—	—
0.1	67	—	—	—
0.5	104	—	—	—
1.0	91	—	—	—
75	92	5	—	—
150	72	8	—	—
270	—[c]	—	—	4
298	83	6	—	—
2012	—	—	7[d]	—

[a] Product of RNA concentration and time (Birnstiel *et al.*, 1972).
[b] L cells infected with A59 virus in the presence of actinomycin D (see Methods).
[c] Not tested.
[d] C_rt calculated using the starting amount of RNA before poly(A) selection (500 μg).

subgenomic RNAs. Taken together, the evidence suggests that coronaviruses, as exemplified by MHV and IBV, code for five to six polyadanylated subgenomic RNAs whose molecular weights lie within the range of approximately 0.8–4 × 10^6. The existence of homologous sequences within a set of viral RNAs suggests parallels with certain other positive-strand RNA viruses. In the replication of the alphavirus, Semliki Forest virus, structural and nonstructural polypeptides are translated from a 26 S RNA and a 42 S RNA, respectively. A copy of 26 S RNA sequence is present within the 42 S RNA molecule, although translation of the 42 S RNA appears to yield only nonstructural polypeptides (Glanville *et al.*, 1976). It has been suggested in studies with Semliki Forest virus (Glanville *et al.*, 1976) as well as certain plant viruses (Shih and Kaesberg, 1973; Hunter *et al.*, 1976) and polyoma virus (Hunter and Gibson, 1978) that translational initiation of eukaryotic mRNAs may occur at only a single site near the 5′ terminus, and that additional coding regions located further downstream are not translated. Thus, the transcription of a set of viral mRNAs which share 3′ end sequence homology but differ in length may be a common effective mechanism to ensure translational initiation of all viral genes by the host cell.

Contemporary analyses of MHV show the presence of three major size classes of structural proteins (Anderson *et al.*, 1979; Bond *et al.*, 1979; Sturman, 1977; Wege *et al.*, 1979) as well as a polyadenylated, single-stranded, RNA genome of approximately 5.4 × 10^6 daltons (Lai and Stohlman, 1978; Wege *et al.*, 1978). Using reported molecular weight estimates for the three major viral structural proteins of A59 (Sturman *et al.*, 1980), i.e., a 180K dalton glycoprotein, a 50K nucleocapsid protein, and a 23K protein, it can be calculated that somewhat less than half of the total coding capacity of the genome is occupied by sequences encoding these structural polypeptides. The remainder of the genome would presumably comprise nonstructural protein-encoding regions as well as any noncoding regions. Experiments performed on the JHM strain of MHV (Siddell *et al.*, 1980) suggest that at least two viral structural polypeptides are encoded by separate mRNAs rather than by a single polycistronic mRNA as in the

case of other positive-strand viruses such as alphaviruses (Mowshowitz, 1973) or enteroviruses such as poliovirus (Jacobson and Baltimore, 1968). Our own studies with MHV (Cheley and Anderson, in press) have shown that each of the three major structural polypeptides is translationally initiated in an independent rather than in a sequential manner; this result is again indicative of the existence of separate viral mRNAs for each structural protein. Assuming, therefore, three mRNAs for structural polypeptides and an approximately equal number for nonstructural proteins, the present finding of six major polyadenylated viral RNAs in MHV-infected cells is not inconsistent with the total number of mRNAs expected. We are currently attempting to verify this idea by characterization of the *in vitro*-directed translation products from each of the subgenomic RNA species.

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