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RNA and Polypeptide Homology among Murine Coronaviruses

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Using a $^{32}$P complementary DNA (cDNA) prepared against the A59 nucleocapsid protein messenger RNA, we have investigated the extent of homology between A59 and four other strains of murine hepatitis virus (MHV). Analysis by hybridization kinetics of the annealing between A59 [$^{32}$P]cDNA and infected cell RNA from the other four MHV strains demonstrated 70-80% homology. By gel transfer analysis, the A59 [$^{32}$P]cDNA was able to detect subgenomic-size virus-specific RNAs in cells infected with all of the five MHV strains. A similar pattern of seven viral RNAs was detected in cells infected with A59, MHV-1, MHV-3, and JHM. In contrast, cells infected with MHV-S contained seven virus-specific RNAs, of which only the two smallest species comigrated with RNAs from the other four strains. The results suggest that as previously shown with A59 (S. Cheley, R. Anderson, M. J. Cupples, E. C. M. Lee Chan, and V. L. Morris (1981) Virology, 112, 596-604), all MHV strains tested encode a nested set of subgenomic RNAs. Analysis of [$^{35}$S]methionine-labeled viral proteins by SDS-polyacrylamide gel electrophoresis revealed that each strain of MHV specified four major viral polypeptides with apparent molecular weights very similar to those previously reported for the E2, N, E1, and P1 polypeptides of A59. The strong degree of interstrain homology among the five MHV strains investigated was confirmed by comparative chymotryptic peptide mapping of the viral N proteins. A majority of the chymotryptic peptides from each of the [$^{35}$S]methionine-labeled N proteins was found to coelute by high-performance liquid chromatography. Moreover, this technique of peptide mapping indicated a particularly strong relatedness between MHV-1 and MHV-S and among MHV-3, JHM, and A59.

INTRODUCTION

Coronaviruses include a number of viral pathogens which are responsible for a diverse spectrum of pathological conditions afflicting rodents (Cheever et al., 1949, Gledhill and Andrews, 1951), fowl (Beaudette and Hudson, 1937), humans (Hamre and Procknow, 1966; McIntosh et al., 1967) as well as several other animal species. Among the coronaviruses pathogenic for rodents, murine hepatitis viruses (MHV) have received considerable attention. Several distinct serotypes of MHV have been recognized (Hierholzer et al., 1979; Piazza, 1969) and numerous studies have centered on the pathological effects of MHV infection of mice and rats. Of particular interest is the JHM strain which, unlike other

MHV strains, shows only a moderate propensity toward visceral tissue, instead, producing a selective infection of the nervous system (Bailey et al., 1949; Cheever et al., 1949; Weiner, 1973; Lucas et al., 1977). Neurological infections by JHM virus can, in some cases, be of a selectively demyelinating nature (Haspel et al., 1978; Nagashima et al., 1978; Sorensen et al., 1980; Weiner, 1973); this feature has prompted analogies between JHM disease and human demyelinating conditions of suspected viral etiology (Weiner et al., 1973). Other strains of MHV produce varying degrees of hepatic disease (Dick et al., 1956; Gledhill and Andrews, 1951; Manaker et al., 1961; Nelson, 1952; Rowe et al., 1963) occasionally with neurological involvement.

Apart from serological relationships (Hierholzer et al., 1979; Piazza, 1969) and preliminary comparisons of viral polypep-
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very little is presently known about structural similarities or differences among MHV strains at the molecular level. We report here the results of comparative homology determinations on virus-specified RNAs and polypeptides of five selected strains of MHV. In conducting the studies on viral RNAs, we have made use of a complementary DNA probe prepared from nucleocapsid (N) protein messenger RNA isolated from cells infected with the A59 strain of MHV. The specificity of this cDNA including its ability to recognize common sequences in A59-specified subgenomic RNAs has been documented elsewhere (Cheley et al., 1981).

MATERIALS AND METHODS

Cell and virus strains. Mouse cell lines, L-2 (Rothfels et al., 1959), LM (Merchant and Hellman, 1962), and 17CL-1 (Sturman and Takemoto, 1972) were used for studies of virus growth. Virus was propagated and plaque assayed (Lucas et al., 1977) in L-2 cells. Virus strains MHV-1, MHV-3, MHV-S, A59, and JHM were obtained from the American Type Culture Collection.

Assays of virus growth. Confluent monolayer cultures of L-2, LM, and 17CL-1 cells in 25-cm² tissue culture flasks (Falcon) were inoculated at a multiplicity of infection (m.o.i.) of 0.5 with one of the five MHV strains. After 1 hr adsorption at 37°C, monolayers were freed from residual inocula by thrice rinsing with minimal essential medium (MEM) and subsequently incubated at 37°C with 5 ml of MEM supplemented with 5% fetal calf serum (FCS). Aliquots were removed from the supernatant media at 4-hourly intervals and quantitated for infectious virus by plaque assay.

Isolation of [35S]methionine labeling of viral polypeptides. Confluent cultures of L-2 cells in 60-mm petri dishes were inoculated at an m.o.i. of 5 each with one of the five MHV strains. Cultures were incubated at 37°C for 6-9 hr until virus-induced fusion was evident in virtually all cells; cultures were subsequently incubated for 30 min with [35S]methionine (100 µCi/ml) in 1 ml methionine-free MEM. Cells were harvested by scraping, spun into pellets at 650g for 10 min, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) as previously described (Cheley and Anderson, 1981).

Peptide mapping by high-performance liquid chromatography (HPLC). Each of the five [35S]methionine-labeled nucleocapsid (N) proteins were excised as slices from dried SDS-polyacrylamide gels after visualization by autoradiography. Proteins were eluted from gel slices with (1 ml) immunoprecipitation (I.P.) buffer (Nusse et al., 1978, but containing 0.2% instead of 0.1% SDS) for 8 hr at room temperature with periodic mixing. Solubilized protein was freed from gel debris by centrifugation at 60,000g for 1 hr in a Beckman SW56 rotor. Supernatants were diluted with 1 vol of I.P. buffer lacking SDS in order to...
attain a final SDS concentration of 0.1% and mixed with 50 μl of anti-MHV antiserum. Immune precipitates were pelleted at 60,000g and digested for 24 hr at room temperature with 1 ml of 0.05 M ammonium bicarbonate containing chymotrypsin (25 μg/ml; Worthington). The resulting peptides were lyophilized, dissolved in application buffer (pyridine:acetic acid:water, 1:1:31), and stored at -20°C under a nitrogen atmosphere prior to analysis. For peptide mapping, a Waters Associates (Milford, Mass.) liquid chromatograph equipped with a Model U6K injector and a Model 6000A solvent delivery system was used. Peptides were eluted from a C18 Bondapak reversed phase column (10 μm, 4 mm × 30 cm) using a phosphoric acid–acetonitrile solvent system (Fullmer and Wasserman, 1979). A linear gradient of 14–36% acetonitrile in 0.1% orthophosphoric acid was found to provide optimal resolution of peptides. Constant flow gradient elution (2 ml/min) was controlled with a Waters Model 720 solvent programmer. Column effluent was fraction collected in volumes of 0.8 ml and radioactivities quantitated by liquid scintillation spectrometry after the addition of Atomlight (New England Nuclear).

RESULTS

Replication of Five Strains of MHV in Selected Cell Lines

Differences in virus replication and virus-induced cytopathic effect (cpe) have been observed in various cell lines infected with MHV (Lucas et al., 1977, 1978). In order to systematically compare the growth characteristics of different strains of MHV, parallel cultures of L-2 (Rothfels et al., 1959), LM (Merchant and Hellman, 1962), and 17CL-1 (Sturman and Takemoto, 1972) cells were each inoculated at an m.o.i. of 0.5 with one of the five strains: MHV-1, MHV-3, A59, or JHM. In all cases, infected L-2 cultures showed the most rapid progression of cpe, in the form of cell–cell fusion which resulted in formation of a virtually complete syncytial monolayer by 12 hr p.i. The light-microscopic appearance of individual L-2 cell syncytia produced by infection with either MHV-1, MHV-3, A59, or JHM was identical to that previously observed in JHM-infected RN2 cells (Lucas et al., 1977). However, L-2 cultures infected with MHV-S produced syncytial foci which tended to contain a large number of predominantly spherical, multinucleated cells which detached from the monolayer more readily than the more flattened syncytia produced by the other four virus strains.

In contrast to infected L-2 cultures, cultures of 17CL-1 cells inoculated with any of the five MHV strains did not show significant cpe until approximately 16 hr p.i., at which time a few isolated syncytia were detectable. By 20 hr p.i. approximately 5–10% of the cell monolayer, as estimated by light microscopic observation, consisted of syncytia. Infected LM cell cultures showed the least cpe of any of the three cell lines tested; by 20 hr p.i. only a very few isolated syncytia were visible in each flask.

Virus replication, as determined by plaque assay of supernatant media at 4-hourly intervals, is shown in Fig. 1. In both L-2 and 17CL-1 cell cultures, maximum titers were reached between 12 and 16 hr p.i. for all five MHV strains tested. Growth of virus in LM cells in contrast was markedly reduced and the growth curves more erratic than those observed in L-2 or 17CL-1 cells. Longer term culturing of LM cells infected with all five MHV strains (unpublished) has shown that virus continues to be produced over several weeks in a similar fashion to that in a number of other cell lines previously shown to support a persistent infection of MHV (Lucas et al., 1978). It is thus interesting that all five MHV strains show the ability to undergo either a productivity lytic (in L-2 cells) or a mildly cytopathic, chronic (in LM cells) type of infection depending on host cell.

Comparison of Murine Coronavirus RNA Using the RNA Transfer Procedure

A complementary DNA (cDNA) was prepared from the MHV A59 0.8 × 10^6-dalton mRNA encoding the nucleocapsid pro-
FIG. 1. Replication of five MHV strains in selected cell lines. Cultures of L-2 (panel A), CL-1 (panel B), and LM (panel C) were inoculated at an m.o.i. of 0.5 with MHV-1 (×), MHV-3 (○), MHV-S (●), A59 (■), or JHM (□). After removal of unadsorbed inocula, cultures were incubated in MEM supplemented with 5% FCS at 37°C. Aliquots were removed from supernatant media for quantitation of infectious virus by plaque assay.

In each case, the A59 cDNA annealed with several RNA bands, indicating significant homology between A59 nucleocapsid protein mRNA and the corresponding sequences in the other strains of MHV. The overall similarity in RNA patterns (Fig. 2) is most striking for strains A59, MHV-1, MHV-3, and JHM; for MHV-S a slightly different pattern was observed in which only the two smallest RNA species comigrated with corresponding RNA species seen in the other four samples. In order to enhance the observed migrational differences between the RNAs of MHV-S and those of the four other murine coronaviruses (as typified by A59), prolonged times of electrophoresis, were used (Fig. 3). The 1.4 \times 10^6- and 1.6 \times 10^6-dalton species were missing from the MHV-S pattern, and a 1.2 \times 10^6-dalton virus-specific RNA species
FIG. 2. Comparison of murine hepatitis virus RNA species using electrophoresis and RNA transfer procedures. RNA was extracted from L-2 cells infected with different strains of MHV. RNA was subjected to electrophoresis in a 1.5% agarose gel (40 V 18-hr). The RNA was then transferred to DBM paper and annealed to A59 [\textsuperscript{32}P]cDNA. Virus-specific RNA was visualized by autoradiography (see Materials and Methods). (A) MHV-S-infected cell RNA (30 μg). (B) MHV-1-infected cell RNA (30 μg). (C) MHV-3-infected cell RNA (30 μg). (D) JHM-infected cell RNA (30 μg). (E) A59-infected cell RNA (30 μg). (F) Uninfected L-2 cell RNA (30 μg). Molecular weights refer to A59 virus-specific RNA present in infected L-2 cells (Cheley et al., 1981).

was present in MHV-S but was absent in A59 (Fig. 3). In addition, MHV-S had three higher-molecular-weight virus-specific RNA species (in 3-4 × 10^6-dalton range) while A59 only had two viral RNA species in this region of the gel. However, the
Comparison of Murine Coronaviruses Using Hybridization Kinetics

We used the cDNA prepared from A59 nucleocapsid mRNA to investigate the homology among the nucleocapsid-encoding sequences of the five different strains of murine Coronavirus. In agreement with our previous results, it was found that the A59 cDNA annealed essentially to completion with A59 infected cell RNA (Cheley et al., 1981; Fig. 4). The other four murine Coronaviruses that were tested annealed to a maximum extent of 70-80% with the A59 cDNA (Fig. 4A-D). The $C_{t_{1/2}}$ values varied from $0.8 \times 10^{-1}$ to $1.6 \times 10^{-1}$; this difference could be due to small discrepancies in the initial multiplicity of infection. All of these $C_{t_{1/2}}$ values correspond to greater than 1000 genome equivalents per infected cell using the calculation method of Varmus et al. (1973). Thus all five murine hepatitis virus strains produced high levels of virus-specific RNA when infecting L-2 cells. As with our previous work (Cheley et al., 1981), the cDNA annealed to less than 8% with uninfected L-2 cell RNA or yeast RNA.

Comparison of $[^{35}S]$Methionine-Labeled Viral Polypeptides from Five MHV Strains

L-2 cells infected with each of the five MHV strains and labeled with $[^{35}S]$-methionine were found, in each case, to contain four major labeled polypeptides which were absent in uninfected cells. As
FIG. 4. Homology of murine hepatitis virus RNA by hybridization kinetics. RNA was extracted from L-2 cells infected with different strains of murine hepatitis virus and annealed with A59 \(^{32}P\)-cDNA (1000 cpm per time point; 2-4 \(\times\) 10^6 cpm/µg; see Materials and Methods). The hybridization curve with A59-infected cell RNA (●) was repeated in each panel for comparison. (A) MHV-1-infected cell RNA (△). (B) MHV-S-infected cell RNA (○). (C) JHM-infected cell RNA (○). (D) MHV-3-infected cell RNA (□).

previously shown for MHV-3 (Anderson et al., 1979; Cheley and Anderson, 1981), JHM (Anderson et al., 1979), and A59 (Cheley et al., 1981) these four polypeptides constitute three major size classes of approximately 180,000, 50,000-56,000, and 20,000-25,000 daltons. The striking similarity in electrophoretic mobilities among the five strains of each of the four major polypeptides is evident from Fig. 5. Nevertheless, slight variations in apparent molecular weight are detectable. Thus, for example, the polypeptides corresponding in size to the nucleocapsid N protein described for A59 (Sturman et al., 1980) have the following approximate molecular weights as deduced from Fig. 5; MHV-1, 56,000; MHV-3, 56,000; MHV-S, 56,000; JHM, 55,000, and A59, 52,000. Although it remains to be demonstrated that similar-sized polypeptides for each MHV strain are functionally and structurally equivalent, peptide mapping data on the N proteins (in the following section) as well as on the remaining viral proteins (unpublished) strongly suggested that this is, in fact, the case. We have, therefore, adopted the nomenclature proposed for A59 by Sturman et al. (1980) to designate the large ca. 180,000-dalton protein as E2 and the small, ca. 24,000-dalton protein as E1, for each of the five MHV strains. As previously shown for MHV-3 (Cheley and Anderson, 1981) E1 is post-translationally derived from PE1. Moreover, preliminary experiments with the other MHV strains suggest that in all cases a precursor-product relationship exists between the corresponding two polypeptides.

It should be noted that several other labeled proteins are present in certain extracts, particularly those of the JHM (Fig.
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Comparative Peptide Mapping of MHV Nucleocapsid N Proteins

Analysis of the chymotrypsin-derived peptides from each of the MHV nucleocapsid N proteins revealed strong overall interstrain similarity and suggested even stronger interrelationships between certain members (Fig. 6). In all five cases, coeluting peptides were evident at fraction numbers 30, 50, 58, 71, and 96. Two of these peptide peaks (fraction numbers 50 and 58) were of much lower relative intensity in both the MHV-1 and MHV-S peptide profiles than with those of the other MHV strains. The reason for this difference in peak intensity is not known. One explanation is that these two peptides from MHV-1 and MHV-S, while chemically similar to corresponding peptides of the other three MHV strains, may be nevertheless somewhat more deficient in methionine, and hence in $[^{35}\text{S}]$-methionine label. Alternatively, steric factors may limit quantitative liberation of these two peptides from MHV-1 and MHV-S-derived N proteins. Either of these explanations may also be applicable to the relatively low intensity of the fraction 58 peptide (relative to that at fraction 50) in the case of the MHV-3 N protein. As can be seen in Fig. 6 the peptide elution profiles for the MHV-1 and MHV-S N proteins were virtually identical, suggesting very strong relatedness between these two viral strains. Among the other three viruses, MHV-3, JHM, and A59, a majority of the N-protein-derived peptides comigrated, namely those around fraction numbers 10, 18, 30, 50, 58, 71, and 96.

Since the migrational characteristics of a peptide may be altered by a single amino acid substitution (Ingram, 1956; Hunt and Ingram, 1959), we interpret the above data as indicating a high degree of interstrain homology among nucleocapsid N proteins of murine coronaviruses.

DISCUSSION

The results of the present study extend our knowledge of murine coronaviruses in the following areas.

1. Our previous finding (Cheley et al., 1981) that cells infected with the A59 strain of MHV contain six or seven virus-coded, subgenomic RNAs, has now been expanded to include four additional strains.
of MHV. As in the case of A59, these subgenomic RNA species contain sequences in common.

2. All five MHV strains tested show a remarkably similar polypeptide profile with only slight variations in apparent molecular weight. Three major size classes of viral polypeptides are evident in each case, which are analogous to those already described for MHV-A59 (Sturman et al., 1980; Cheley et al., 1981).

3. Strains MHV-1, MHV-3, MHV-S, and JHM have a high degree of nucleic acid homology (70–80%) with the A59 strain as judged by hybridization with labeled cDNA prepared from A59 nucleocapsid protein-encoding mRNA. A further substantiation of strong homology among MHV strains within this coding region was obtained by comparative peptide mapping of each of the virus-coded nucleocapsid polypeptides.

The difference in the pattern of virus-specified subgenomic RNAs, between MHV-S and the other four MHV strains is intriguing. Comigration is observed, among all five strains, for the two smallest RNAs (0.8 × 10^6 and 1.1 × 10^6 daltons); however all higher-molecular-weight RNA species of MHV-S fail to line up with the larger RNAs specified by the other four MHV strains. It is obvious from the present study as well as from our previous work (Cheley et al., 1981) that MHV-coded subgenomic RNAs contain common sequences. It has been demonstrated with the avian coronavirus, infectious bronchitis virus (Stern and Kennedy, 1980), that virus-specific, subgenomic RNAs may form a nested set with sequence homology at the 3' ends. Assuming a similar situation exists among murine coronaviruses, any alteration of a single subgenomic RNA species would also be present in all larger subgenomic RNA species. Thus, the atypical subgenomic RNA pattern exhibited by MHV-S may reflect genetic alteration at the third smallest subgenomic RNA (i.e., the smallest RNA species which shows altered electrophoretic mobility from the corresponding RNA of the other four MHV strains). It is interesting to note, nevertheless, that any such alteration is likely to have occurred within the structural protein-encoding regions of MHV-S RNA, since all three major structural MHV-S proteins show very little deviation in apparent molecular weight from those of the other MHV strains (Fig. 5).

Homology comparisons of different virus strains have been performed on several virus genera including orthomyxoviruses (Erickson and Kilbourne, 1980; Laver and Downie, 1976; Shild et al., 1979; Scholtissek et al., 1976), rhabdoviruses (Burge and Huang, 1979; Doel and Brown, 1978; Repik et al., 1974), and retroviruses (Albino et al., 1979; Barbacid et al., 1977; Gautsch et al., 1978; Oroszlan et al., 1975). From a survey of such studies, it is evident that genetic variation between related strains can involve changes at any sub-
genomic site. There may, however, be a relatively greater degree of conservation within regions encoding internal virion polypeptides than within those coding for surface polypeptides. In rationalizing this observation it has been suggested (Doel and Brown, 1978; Laver and Downie, 1976) that sequence conservation among internal virion polypeptides may reflect stringent structural constraints imposed on such polypeptides by the precise nature of their interactions during virus assembly. Thus, in the case of MHV, in which the major internal protein is the nucleocapsid polypeptide (Struman et al., 1980), one might expect that this polypeptide be fairly conserved among various strains of MHV. Indeed, the results of the present study demonstrate strong relatedness of all five MHV isolates in both the N polypeptide as well as its corresponding RNA coding sequence. Studies are presently in progress to determine homology relationships of the remaining two major virion structural polypeptides E1 and E2.

A logical extension of the present study is to attempt to precisely define the sites of genetic variability among the currently recognized strains of MHV. To date considerable differences in virulence and tissue tropism have been demonstrated. JHM, alone among the five MHV strains used in the present work, has been shown to produce a highly selective infection of the central nervous system (Bailey et al., 1979; Cheever et al., 1949; Weiner, 1973). Of the other four MHV strains, which generally produce liver disease with occasional neurological involvement, strains MHV-1 (Dick et al., 1956; Gledhill and Andrewes, 1951) and MHV-S (Rowe et al., 1963; Taguchi et al., 1980) appear to be of relatively low pathogenicity. While disease outcome may be a function of host strain (Bang and Warwick, 1960), host age (Biggers et al., 1964, Dick et al., 1956; Weiner, 1973), antiviral factors (Virelizier and Gresser, 1978), and passage history of the virus (Rowe et al., 1963; Weiner, 1973), the underlying differences in biological virulence of varying MHV strains have been shown to reflect primarily virus-specific determinants (Dick et al., 1956; Lucas et al., 1977; Haspel et al., 1978). The results of the present study suggest a high degree of relatedness among different MHV strains and thus provide hope that such determinants may eventually be identified.

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