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Coronaviruses SD and SK Share Extensive Nucleotide Homology with Murine Coronavirus MHV-A59, more than That Shared between Human and Murine Coronaviruses

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A cDNA probe representing the genome of mouse hepatitis virus (MHV) strain A59 (MHV-A59) was used to measure nucleotide sequence homologies among murine and human coronaviruses and the SD and SK coronaviruses isolated by Burks *et al.* Since SD and SK were isolated by inoculation of multiple sclerosis (MS) central nervous system (CNS) tissue into mice or cultured mouse cells, it is important to determine their relationships to other murine and human coronavirus isolates. Our results indicate that SD and SK share almost complete nucleotide homology (approximately 90%) with MHV-A59 and generate subgenomic RNAs of the same sizes as MHV-A59. The human coronavirus (HCV) strains tested show less homology with MHV-A59. The immunologically unrelated HCV-229E shows no nucleotide homology with MHV-A59. The immunologically cross-reactive HCV-OC43 shows nucleotide homology with MHV-A59 by blot hybridization but not when hybridized in solution and assayed by S1 nuclease digestion.

INTRODUCTION

Coronaviruses have been associated with acute and chronic neurological diseases in many species of animals (McIntosh, 1974). Infection of rodents with the murine coronavirus, mouse hepatitis virus MHV strain JHM, has been used as a model system to study virus-induced demyelination (Weiner, 1973; Nagashima *et al.*, 1978; Stohlman and Weiner, 1981). After initial panencephalitis caused by MHV-JHM, this virus produces a persistent infection with primary demyelination with some evidence for remyelination (Weiner, 1973). Thus persistent MHV-JHM infection of rodents has been cited as a model to study the human demyelinating disease multiple sclerosis (MS).

Human coronaviruses (HCV) are ubiquitious in nature with a large portion of the human population possessing neutralizing antibodies (McIntosh, 1974). These viruses were isolated usually as respiratory, and occasionally as enteric viruses. They are estimated to be responsible for 15% of common colds (McIntosh, 1974). There are no reports thus far of involvement of human coronaviruses with persistent neurological disease. Some strains of HCV such as OC43, are antigenically related to murine coronaviruses such as MHV strain JHM (McIntosh, 1974; Gerdes *et al.*, 1981a, b) and may be grown in the brains of suckling mice (McIntosh *et al.*, 1967). Others such as HCV-229E are unrelated antigenically to MHV or HCV-OC43 (McIntosh, 1974; Pederson *et al.*, 1978).

Because (1) murine coronaviruses are associated with chronic demyelinating disease in rodents (Weiner 1973; Nagashima *et al.*, 1978), (2) antibody against HCV is very common in the human population (McIntosh, 1974), and (3) there is evidence suggesting that MS may be caused by a virus, various workers have undertaken comparisons of human and murine coronaviruses and have started to search for coronaviruses in central nervous system (CNS) tissue from MS patients. There is one report of particles with coronavirus-

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like morphology seen by electron microscopy in brain tissue from an MS patient (Tanaka *et al.*, 1976). More recently Burks and co-workers have isolated two coronaviruses, designated SD and SK, by intracerebral inoculation of unfrozen MS CNS autopsy tissue either into weanling mice or into 17CL-1 mouse cells in culture (Burks *et al.*, 1980). Gerdes *et al.* (1981a, b) and we (this manuscript) have examined the relationship between these viruses and other known murine and human coronaviruses. Gerdes *et al.* (1981a, b) showed that SD and SK are antigenically related to MHV-A59 and to HCV-OC43 but not HCV-229E. They were inconclusive about which strains their isolates were more related to. We have further compared murine and human coronaviruses and SD and SK by using molecular hybridization of virus-specific RNA with cDNA probes. Our results show extensive nucleotide homology between SD and SK and MHV-A59, more than that between the human viruses and MHV-A59.

MATERIALS AND METHODS

Viruses and cells. MHV-A59 was grown in 17CL-1 cells as previously described (Weiss and Leibowitz, 1983). SD and SK viruses (Burks *et al.*, 1980) were obtained from Dr. J. Gerdes and were also grown in 17CL-1 cells. HCV-229E was obtained from the American Type Culture Collection (ATCC) and grown in human embryonic lung (L-132) cells also obtained from the ATCC. These viruses were plaque purified two times and grown in Dulbecco's medium with 10% fetal calf serum (Robb and Bond, 1979).

HCV-OC43 was obtained as a 20% suckling mouse brain suspension from Dr. J. Hierholzer at the Center for Disease Control (CDC), Atlanta. It was inoculated intracerebrally into C57BL/6 suckling mice, harvested 2 days later, and a 10% brain suspension was made in phosphate-buffered saline (PBS) containing 0.75% bovine serum albumen. The mothers of the suckling mice were obtained from Jackson Labs as MHV-free animals. All were negative for antibodies against MHV-A59 (as determined by an enzyme-linked immunosorbent assay) and HCV-OC43 (as shown by a lack of OC43 hemagglutination inhibiting activity in the sera of these animals (Hierholzer *et al.*, 1969)) and thus were considered uninfected by these coronaviruses. Virus in brain homogenates was assayed by hemagglutination of chicken red blood cells at room temperature (Hierholzer *et al.*, 1969). Virus was further verified as HCV-OC43 since hemagglutination was inhibited by an anti-OC43 reference antisera obtained from CDC. HCV-OC43 was also grown in human rectal tumor (HRT) cells (Laporte *et al.*, 1980) obtained from Dr. David Brian. Infected mouse brain homogenates were adsorbed onto monolayers of HRT cells for 1 hr at room temperature. The cells were extensively washed, medium added, and the cells incubated at 33°. Virus in the supernatant was titered at various times postinfection by hemagglutination (Hierholzer *et al.*, 1969). Mock-infected cells were adsorbed with a brain homogenate from uninfected suckling mice.

cDNA probes. cDNAs were synthesized using purified genome RNA as template, oligomers of calf thymus DNA as primers, and reverse transcriptase (Taylor *et al.*, 1976). cDNAs were labeled with [^{32}P]dCTP to specific activity of approximately 10^8 cpm/μg. When used for liquid hybridization, cDNA was synthesized in the presence of actinomycin D and was >95% single stranded. Such cDNAs were validated to be highly virus specific and to represent the majority of the genome RNA as previously described in detail (Weiss and Leibowitz, 1981, 1983).

RNA extraction. MHV-A59, SD, SK, and HCV-229E virus infections were carried out with a multiplicity of infection of between 0.1 and 1 plaque-forming units per cell. RNA was extracted 18 hr after infection with A59, SD, and SK viruses, when massive syncytia were present. 229E-infected cells were labeled with [^{3}H]uridine in the presence of 10 μg/ml actinomycin D from 18 to 24 hr postinfection when RNA was extracted. RNA was extracted from OC43-infected HRT cells at 3 days post-

infection. RNA was extracted from the cytoplasm of infected cells by SDS-proteinase K treatment followed by phenol extraction as previously described (Weiss and Leibowitz, 1983). RNA was extracted from suckling mouse brain homogenates by SDS-proteinase K treatment followed by phenol extraction (Weiss, Varmus and Bishop, 1977).

RNA analysis. (1) Gel electrophoresis. RNA was electrophoresed in 1% agarose gels, in the presence of methyl mercury hydroxide (Bailey and Davidson, 1976) or formaldehyde (Lehrach *et al.*, 1977) as denaturant. Gels were either fluorographed with sodium salicylate (Chamberlain, 1979) or blotted onto nitrocellulose (Thomas, 1980). (2) Blots. Dot blots. RNA was adsorbed onto nitrocellulose filters in various amounts (as designated in figure legends), dried, and the filters were baked and hybridized with cDNA (Thomas, 1980). Northern blots: RNA was electrophoresed in gels, blotted onto nitrocellulose filters, and hybridized with cDNA (Alwine *et al.*, 1977; Thomas, 1980). (3) Hybridization in solution was carried out at 68°, 0.6 *M* NaCl, and assayed by S1 nuclease digestion as previously described (Leong *et al.*, 1972). In C_rt curves, increasing amounts of RNA were hybridized with a fixed amount of cDNA to achieve increasing C_rt values where C_rt = concentration of RNA × time of hybridization.

RESULTS

OC43 RNA

HCV-OC43 has been difficult to grow and assay in cell culture and this has impaired the study of viral nucleic acids. This virus is usually grown in the brains of suckling mice and titered either by infection of suckling mice or by hemagglutination (Hierholzer *et al.*, 1969). Schmidt *et al.* (1979) have reported growing and plaquing HCV-OC43 on human rhabdomyosarcoma (RD) cells. Although we had difficulty with growing the virus in RD cells, we have had some success with growth in human rectal tumor (HRT) cells (Laporte *et al.*, 1980). We have used hemagglutination to detect and quantitate HCV-OC43 in both infected suckling mouse brain homogenates and HRT cell supernatants. As shown in Table 1 brain homogenates from infected mice contained 40,000 HAU/ml of OC43 and homogenates from control mock-infected animals had none. This activity could be specifically inhibited by anti-OC43 reference antisera but not by A59 antisera or preimmune sera (data not shown).

Also shown in Table 1 after infection of HRT cells with OC43-infected mouse brain homogenates (800 hemagglutinating units/10^6 cells), hemagglutinating activity could be measured in the medium. As expected pretreatment of brain homogenates with antisera directed against OC43 prevented

TABLE 1

TITERS OF HCV-OV43 IN SUCKLING MOUSE BRAIN HOMOGENATES AND HRT CELL SUPERNATANTS

Homogenate or supernatant	HAU/ml[a]
OC43-infected suckling mouse brain homogenate	40,000
Mock-infected suckling mouse brain homogenate[b]	0
OC43-infected HRT cells	
1 day postinfection	0
2 days postinfection	0
3 days postinfection	80
4 days postinfection	1,280
5 days postinfection	5,120
Mock-infected HRT cells[b]	
5 days postinfection	0
HRT cells infected with antibody-treated OC43[c]	
5 days postinfection	0

[a] Hemagglutinating units/ml of OC43. One HAU is defined as the amount of virus present in 0.05 ml of the highest dilution of brain homogenate or supernatant capable of agglutinating 0.05 ml of 0.5% chicken erythrocytes in the standard assay described by Hierholzer *et al.* (1969).

[b] Mock-infected mice or cells are mock-infected with a 10% homogenate or uninfected suckling mouse brains.

[c] OC43-infected suckling mouse brain homogenate was incubated with anti-OC43 reference antiserum for 1 hr at room temperature before infecting cells.

growth of the virus. We have used brain lysates of infected mice and infected HRT cells as sources of HCV-OC43 RNA for the experiments described below.

Nucleotide Sequence Homologies among Coronavirus Strains

Figure 1 illustrates the use of dot blot hybridizations to detect nucleotide sequence homologies between MHV-A59 and various murine and human coronavirus strains including the SD and SK putative "MS isolate" strains of Burks *et al.* (1980). A complementary DNA (cDNA) probe representing the majority of sequences of genome RNA of MHV-A59 (Weiss and Leibowitz, 1981, 1983) hybridized to RNA extracted from cells infected with MHV-JHM, HCV-OC43, SD, SK, and from brain homogenates of suckling mice infected with OC43 as well as to its homologous RNA. There was no hybridization detected between A59 cDNA and RNA from cells infected with HCV-229E. Since there are reports that the nucleocapsid protein of HCV-229E has some antigenic cross-reactivity with the other viruses (Gerdes *et al.*, 1981b), the reciprocal experiment using HCV-229E cDNA was also carried out. As illustrated in Fig. 2, cDNA representing the HCV-229E genome hybridized only to its homologous RNA and not to HCV-OC43 RNA or to MHV-A59 RNA.

Quantitation of Homology

The blot experiments illustrated in the above sections show the homology between the nucleic acids of coronavirus strains MHV-A59 and SD, SK, and HCV-OC43 and the lack of homology with HCV-229E. These techniques, however, do not quantitate the percentage of the genome sequences that are homologous. To be more quantitative, solution hybridization experiments were carried out and the extent of hybridization was measured by resistance of hybridized cDNA to S1 nuclease digestion. RNA extracted from cells infected with MHV-A59 or SK was hybridized to single-stranded [^{32}P]cDNA representing MHV-A59. Hybridization was carried out with varying amounts of RNAs to achieve the C_rt values shown in Fig. 3. Hybridization of A59 cDNA to SK RNA was almost as extensive as to its homologous A59 RNA. (The shift in the SK curve to higher C_rt values indicates less virus-specific RNA in the SK-infected cells relative

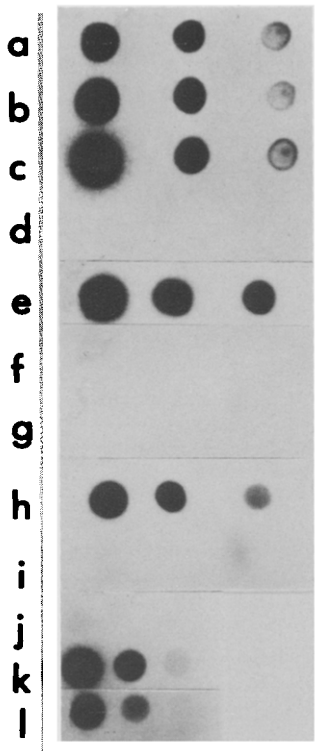

FIG. 1. Dot blot hybridization of MHV-A59 cDNA with coronavirus RNAs. RNA from infected or mock-infected cells or mouse brain homogenates was spotted onto nitrocellulose filters. In the case of intracellular RNAs, 5.0, 0.5, and 0.05 μg amounts were used. In the case of purified genome RNA, 0.1, 0.01, and 0.001 μg were used. Filters were hybridized to 10^6 cpm (10^8 cpm/μg) of A59 [^{32}P]cDNA and autoradiographed (Alwine *et al.*, 1977; Thomas, 1980). (a) SD-infected 17CL-1 cellular RNA. (b) SK-infected 17CL-1 cellular RNA. (c) A59-infected 17CL-1 cellular RNA. (d) Uninfected 17CL-1 cellular RNA. (e) A59-infected 17CL-1 cellular RNA. (f) 229E-infected L-132 cellular RNA. (g) Uninfected L-132 cellular RNA. (h) OC43-infected suckling mouse brain homogenate RNA. (i) Mock-infected suckling mouse brain homogenate RNA. (j) Mock-infected HRT cellular RNA. (k) OC43-infected HRT cellular RNA. (l) A59 purified genome RNA. Lanes a–d, e–i, and j–l are from separate experiments.

to MHV-A59.) Uninfected cell RNA, as expected, showed no hybridization.

Similar hybridizations were carried out with RNAs from cells infected with the other viruses and the plateau values for the percentage cDNA hybridized are summarized in Table 2. SD and SK are almost completely homologous to MHV-A59, more so than another MHV strain, JHM. HCV-229E showed no homology with A59 cDNA as predicted from the blot experiments. HCV-OC43 showed no homology with the cDNA by this assay. This is probably due to stringency of hybridization and S1 nuclease assay for hybridization (see Discussion).

Intracellular RNAs

To further compare the RNA of the murine and human strains the intracellular subgenomic RNAs were examined by gel electrophoresis. As illustrated in Fig. 4, cells infected with MHV-A59 contain six major subgenomic RNAs as well as genome-sized RNA (band 1) (Cheley *et al.*, 1981a; Lai *et al.*, 1981; Leibowitz *et al.*, 1981; Spaan *et al.*, 1981). These range in molecular weight from 0.63×10^6 daltons for RNA 7 to 6.1×10^6 daltons for RNA 1. Intracellular RNAs extracted from cells infected with SD or SK, were electrophoresed in parallel with MHV-A59 RNA, blotted onto nitrocellulose and the virus-specific species detected by hybridization with MHV-A59 cDNA. Seven RNA bands were observed, all comigrating with the major MHV-A59 RNAs. RNAs 2, 4, 5, and, in the case of SK, RNA 3, are less abundant than with MHV-A59 and the extra band between RNAs 2 and 3 is more prominent. The extra bands between RNAs 3 and 4 are also more prominent in the SD RNA.

The intracellular RNAs of HCV-229E were also compared to those of MHV. (In this experiment MHV-3 RNA was used instead of MHV-A59. The genome of MHV-3 is 95% homologous to MHV-A59 and MHV-3 generates the same size intracellular RNAs as MHV-A59 (Cheley *et al.*, 1981b; Weiss and Leibowitz, 1981)). Since HCV-229E RNA does not cross-hybridize with A59 cDNA, 229E intracellular RNA was labeled with [^{3}H]uridine in the presence of actinomycin D (to inhibit host DNA-dependent RNA synthesis). As shown in Fig. 5, 229E also generates six subgenomic RNAs, but they are of different sizes from MHV-3 and hence from SD and SK. (Genome RNA was difficult to observe in this experiment probably due to some RNA degradation).

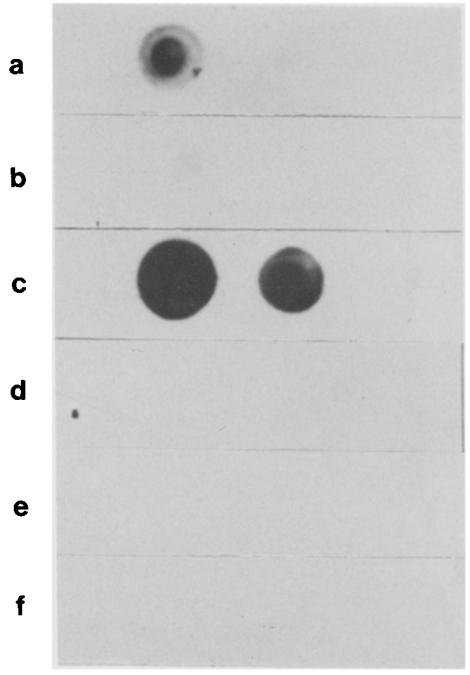

FIG. 2. Dot blot hybridizations of HCV-229E cDNA with coronavirus RNAs. RNA from infected or mock-infected cells or brain homogenates or from purified virions was spotted onto a nitrocellulose filter. In the case of intracellular RNA, 5.0, 0.5, and 0.05 μg were used and in the case of purified genome RNA 0.1, 0.01, and 0.001 μg were used. The filter was hybridized with 10^6 cpm (10^8 cpm/μg) of 229E [^{32}P]cDNA and autoradiographed (Alwine *et al.*, 1977; Thomas, 1980). (a) 229E purified genome RNA. (b) Uninfected L-132 cellular RNA. (c) 229E-infected L-132 cellular RNA. (d) OC43-infected suckling mouse brain homogenate RNA. (e) Mock-infected suckling mouse brain homogenate RNA. (f) A59-infected 17CL-1 cellular RNA.

DISCUSSION

Mammalian coronaviruses have been divided into two antigenic groups (Pederson *et al.*, 1978). One includes MHV, HCV-OC43,

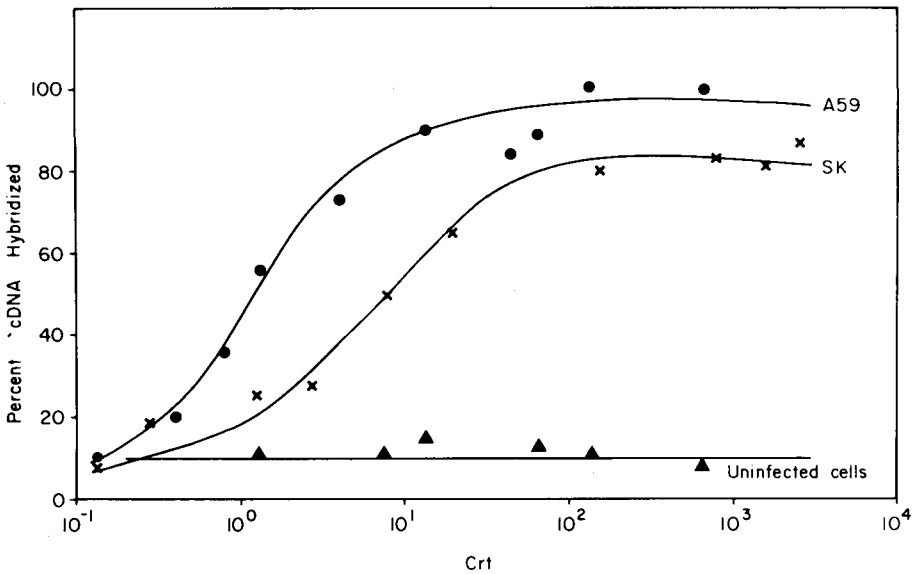

FIG. 3. Kinetics of hybridization of A59 cDNA with cytoplasmic RNA from A59- and SK-infected 17CL-1 cells. Various amounts (80 μg to 8 ng) of intracellular RNA were hybridized with 2000 cpm (10^8 cpm/μg) of A59 [^{32}P]cDNA to the indicated C_rt values. Hybridization was assayed by digestion with S1 nuclease. (●) A59-infected cells. (×) SK-infected cells. (▲) Uninfected cells.

calf diarrhea coronavirus, and hemagglutinating encephomyelitis virus of swine. The other includes HCV-229E, feline infectious peritonitis virus, transmissible gastroenteritis virus of swine, and canine coronavirus. Gerdes *et al.* (1981a, b) have shown that the SD and SK viruses fall into the first group. They showed that all A59 intracellular proteins are immunoprecipitable with antisera directed against SD, SK, or HCV-OC43. From these experiments, however, it was impossible to determine whether SD and SK were more related to human (OC43) or murine (A59) viruses. This is important in determining the origin of SD and SK and the possible link to MS.

TABLE 2

HOMOLOGY AMONG CORONAVIRUS GENOMES[a]

Source of RNA	Percentage hybridization of A59 cDNA
A59 purified virions	100
JHM purified virions	74
229E-infected L-132 cells	0
OC43-infected suckling mouse brain homogenate	0
SD-infected 17CL-1 cells	90
SK-infected 17CL-1 cells	90

[a] RNA from virions, infected cells, or brain homogenates was hybridized with 2000 cpm (10^8 cpm/μg) of A59 [^{32}P]cDNA to completion, the plateau portion of a C_rt curve. Hybridization was assayed by S1 nuclease digestion. These values have been normalized to 100% hybridization of A59 cDNA with its homologous A59 RNA. The actual values of hybridization of A59 cDNA with A59 RNA ranged from 85 to 100%.

We have used nucleic acid hybridization with cDNA to further explore the relationship among these viruses. Our cDNAs are representative of most if not all of the genome RNA sequences (Weiss and Leibowitz, 1981, 1983) and thus are appropriate reagents for quantitating sequence homologies. The relationship between SD and SK and MHV-A59 as determined by molecular hybridization experiments basically agrees with immunological studies. The SD and SK viruses show extensive homology in nucleotide sequence (approximately 90%) to the A59 strain of MHV even when assayed by the stringent S1 nu-

clease assay. This is more homology than that shared between MHV-A59 and another MHV strain, JHM (Lai and Stohlman, 1981; Weiss and Leibowitz, 1981, 1983). Furthermore, the pattern of intracellular RNAs generated by SK and SD is very similar to that of MHV-A59. This is not surprising, considering that Gerdes *et al.*, (1981a, b) showed that cells infected by SD or SK have patterns of viral proteins similar to A59-infected cells (Gerdes *et al.*, 1981a, b). An extra polypeptide of 42,000 daltons molecular weight was observed in

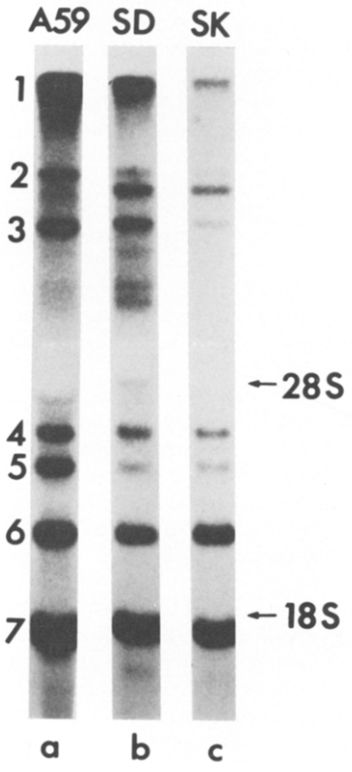

FIG. 4. Comparison of MHV-A59, SD, and SK intracellular RNAs. RNAs (10 μg/lane) extracted from 17CL-1 cells infected with MHV-A59 (lane a), SD (lane b), or SK (lane c) were electrophoresed in a 1% agarose gel containing formaldehyde as a denaturant (Lehrach *et al.*, 1977). After electrophoresis the gel was stained with ethidium bromide to locate ribosomal RNAs, blotted onto nitrocellulose (Thomas, 1980), hybridized with 10^6 cpm of A59 [^{32}P]cDNA, and autoradiographed (Alwine *et al.*, 1977). Arrows designate the position of 18 S and 28 S ribosomal RNAs. Viral RNAs are numbered according to Leibowitz *et al.*, (1981).

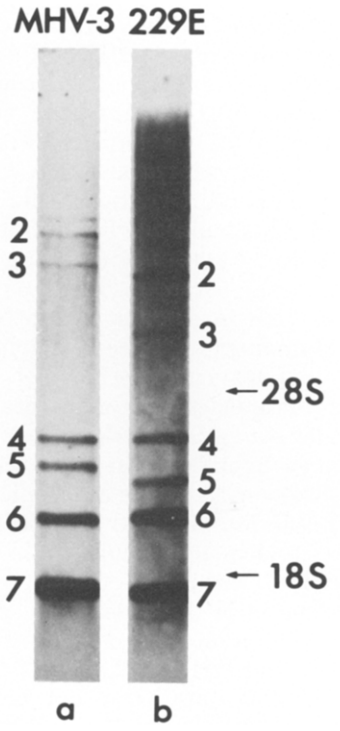

FIG. 5. Comparison of MHV-3 and HCV-229E intracellular RNAs. RNAs (10 μg/lane) extracted from [^{3}H]uridine-labeled MHV-3-infected 17CL-1 cells (lane a) and [^{3}H]uridine-labeled 229E-infected L-132 cells (lane b) were electrophoresed in a 1% agarose gel containing methyl mercury hydroxide as a denaturant (Bailey and Davidson, 1976). After electrophoresis the gel was stained with ethidium bromide to locate ribosomal RNAs and fluorographed (Chamberlain *et al.*, 1979). The positions of 18 S and 28 S ribosomal RNAs are designated by arrows.

SK-infected cells. There is no extra major RNA band seen in the SD and SK samples. The molar ratios of some of the RNAs are different in SD- and SK-infected cells, specifically, RNAs 2 and 5 are less prominent and the extra RNA band between 2 and 3 is quite prominent. The extra bands between RNAs 3 and 4 are also more prominent in the SD sample. However, there is similar variation among the MHV strains (Cheley *et al.*, 1981b; Leibowitz *et al.*, 1981; Weiss and Leibowitz, 1981) and we do not understand its significance. Perhaps this is due to a small amount of degradation in the extraction of these very large RNAs.

Comparison of the human coronaviruses

OC43 and 229E with MHV-A59 reveals less homology than between MHV-A59 and SK and SD. RNA extracted either from brain homogenates of OC43-infected suckling mice or from HRT cells infected with OC43 shows homology with A59 cDNA when assayed by blot hybridization. This homology is not detected when liquid hybridization followed by the more stringent S1 nuclease assay is used. There is precedence for this in at least two other systems. Homology between murine and human papovaviruses (Howley *et al.*, 1979) and murine and human rotaviruses (Schroeder *et al.*, 1982) is detected only under relaxed hybridization and assay conditions. These viruses are antigenically related; this immunological cross-reactivity is detected without difficulty as is the immunological cross-reactivity between MHV-A59 and HCV-OC43.

There is no homology detected between HCV-229E and MHV-A59 or HCV-OC43 in cross-hybridizations using dot blots and cDNA representing either virus. It is unlikely that there is homology betwen HCV-229E and SD and SK since the latter viruses are so closely related to MHV-A59. This is not surprising considering these viruses fall into different antigenic groups (Pederson *et al.*, 1978). However, there are reports of immunological cross-reactivity between the nucleocapsid proteins of these viruses (Gerdes *et al.*, 1981b). These sequences if related, are probably too diverged to be detected by our assay. Perhaps under less stringent blotting conditions, homology could be detected.

These results show that SD and SK are more closely related to MHV-A59 than are the human viruses OC43 and 229E. Because SD and SK are almost completely homologous (90%) to MHV-A59 it is unlikely that they share much homology with either HCV-OC43 or HCV-229E. It is possible that the 10% of SD and SK sequences that are not homologous to MHV-A59 are homologous to either HCV-OC43 or HCV-229E. However, if that were the case, it would still suggest that these isolates are more related to murine than human coronaviruses. This, in addition to the fact that SD and SK grow only in murine and not human cells (Gerdes *et al.*, 1981b), suggests that these viruses are not of human, but murine origin.

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