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Analysis of Genomic and Intracellular Viral RNAs of Small Plaque Mutants of Mouse Hepatitis Virus, JHM Strain

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The genomic RNA and intracellular RNA of mouse hepatitis virus, strain JHM (MHV-JHM) and two plaque mutants (1a and 2c), which have been isolated from a persistently infected culture (JHM-CC), have been analyzed by T1-resistant oligonucleotide fingerprinting. The genomic RNA of the virus population (JHM-CC virus) released from different passage levels of the same persistent infection has also been analyzed. The analysis shows the locations within the genomic and intracellular RNAs of more than 45 T1-resistant oligonucleotides and confirm earlier studies (J. L. Leibowitz, K. C. Wilhelmsen, and C. W. Bond (1981), virology 114, 39-51), showing that the six subgenomic RNAs of MHV-JHM form a 3' coterminal nested set which extends for different lengths in a 5' direction. The analysis also identifies in each subgenomic RNA those large T1 oligonucleotides derived from noncontiguous regions of the genome during mRNA synthesis. Two important conclusions can be reached from analysis of the mutant viruses. First, the virus population released from the persistent infection represents a fairly constant mixture of viruses, and the fluctuating emergence of variants as predominant species in the culture does not occur. Second, the data indicate that for particular intracellular RNAs of mutant viruses the sequence rearrangements occurring during subgenomic mRNA synthesis are different from those in the corresponding intracellular RNA of wild-type virus. The result may indicate a potential flexibility in the leader/body fusion process that has not been previously recognized. © 1984 Academic Press, Inc.

INTRODUCTION

Coronaviruses infect a variety of vertebrates, including man, and are responsible for disease of clinical and economic importance (Siddell et al., 1983). The most studied member of the group is mouse hepatitis virus (MHV) which has a single, positive-stranded genomic RNA of 5.4-6.7 × 10⁶ molecular weight (Lai and Stohlman, 1978; Leibowitz et al., 1981; Spaan et al., 1981; Wege et al., 1978, 1981; Yogo et al., 1977). In cells infected with MHV six species of subgenomic mRNA, together with a genomic-size mRNA, are produced (and are numbered in order of decreasing size). These mRNAs form a 3' coterminal "nested" set but each has at its 5' terminus a common leader, derived from sequences at the 5' end of the genome (Baric et al., 1983; Cheley et al., 1981; Lai et al., 1983b; Spaan et al., 1982, 1983). As MHV replicates in the cytoplasm (Brayton et al., 1981; Wilhelmsen et al., 1981) and mRNA synthesis does not involve the processing of larger precursor RNAs (Jacobs et al., 1981), coronaviruses appear to have evolved a novel mechanism of mRNA synthesis involving the cytoplasmic fusion of noncontiguous transcripts (Spaan et al., 1983). In the infected cell the synthesis of
viral polypeptides is initiated independently (Siddell, 1982, 1983) and in vitro and in vivo translation studies have shown that mRNA7, mRNA6, and mRNA3 encode the virion nucleocapsid protein (NP), the matrix protein (E1), and the precursor of the peplomer protein (E2), respectively (Leibowitz et al., 1982; Rottier et al., 1981b; Siddell et al., 1980; Siddell, 1983).

One MHV strain, MHV-JHM, is of particular interest in that it has the ability to induce central nervous system disorders in mice and rats and can be used as a model for virus-induced demyelination (Herndon et al., 1975; Knobler et al., 1981; Nagashima et al., 1978). Previously, we have established an in vitro persistent infection with MHV-JHM in a DBT cell line (JHM-CC), and found that the virus population released from this culture (JHM-CC virus) formed small plaques on DBT cells and possessed lower virulence to ICR mice when compared with original MHV-JHM (Hirano et al., 1981; Makino et al., 1982). Histopathological examination revealed that after infection with JHM-CC virus, the acute encephalitis characteristic of infection with wild-type virus was absent and instead sharply delineated demyelinating lesions were produced in the brain and spinal cord. The JHM-CC virus will therefore be very useful for studying the pathogenesis of demyelination. In our initial studies (Makino et al., 1983) we have characterized the virological features of two small plaque mutant viruses, 1a and 2c, which were isolated from the JHM-CC virus population. In contrast to wild-type MHV-JHM these mutants grew more slowly, produced no prominent cell fusion during infection, and possessed different amounts and molecular-weight forms of the virion peplomer protein E2.

In this report we have analyzed the genomic and intracellular subgenomic RNAs of MHV-JHM, 1a and 2c, as well as the genomic RNA of the JHM-CC virus population at different passage levels of the persistent culture. These studies indicated that the population of JHM-CC viruses represents a fairly constant mixture of viruses and that different variants do not emerge to predominate during the persistent infection. In some of the subgenomic RNAs of mutant viruses noncontiguous transcripts appeared to have been combined which contain different sequences compared to wild-type MHV-JHM.

MATERIALS AND METHODS

Cell culture and viruses. MHV-JHM, 1a, and 2c viruses were grown on DBT cells as described previously (Makino et al., 1983). Viruses from the persistent culture JHM-CC were harvested at different passage levels and propagated once on DBT cells before use.

Preparation of virion RNA. ³²P-labeled RNA was extracted from purified viruses by procedures described previously (Makino et al., 1983, 1984). In some experiments, the 60 S virion RNA was obtained by separation on a 15-30% sucrose gradient in a SW50.1 rotor at 45,000 rpm for 1.5 hr.

Preparation of intracellular viral RNA. DBT cells were inoculated with MHV-JHM at a multiplicity of 1.0. After adsorption for 1 hr at 37°, cultures were incubated for 2.5 hr with Eagle's minimal essential medium (MEM, Nissui, Tokyo) containing 5% dialyzed calf serum. Then, cultures were incubated with MEM containing 5% dialyzed calf serum, 1/10 normal concentration of phosphate, and 2.5 µg of actinomycin D (AMD)/ml (Sigma, St. Louis, Mo.) (1/10P-medium). At 6 hr postinfection (p.i.) the culture medium was replaced with phosphate-free MEM containing 5% dialyzed calf serum, 1/10 normal concentration of phosphate, and 2.5 µg of AMD, and 250 µCi/ml of ³²P (Japan Radioisotope Association) (³²P-medium). Intracellular viral RNA was extracted at 9 to 10 hr p.i. For the labeling of the intracellular RNA of the mutant viruses, 1a and 2c, the culture medium was replaced at 9 hr p.i. with 1/10P-medium. After 12 hr of infection, cultures were incubated with ³²P-medium and viral RNA was extracted at 24 hr p.i. For the extraction of intracellular viral RNA, monolayers of infected cells were chilled on ice, and washed three times with chilled phosphate-buffered saline, pH 7.2. Then the
cells were scraped by a rubber policeman and centrifuged at 3000 rpm for 10 min at 4°C. The cells were lysed in NTE buffer (0.1 M NaCl, 0.01 M Tris- HCl, pH 7.2, and 1 mM EDTA) containing 0.5% NP-40. After centrifugation at 3000 rpm for 15 min at 4°C to remove nuclei, the RNA was isolated from the supernatant following phenol/chloroform extractions as described previously (Makino et al., 1984).

**Oligo(dT)-cellulose column chromatography.** Ethanol-precipitated RNA was dissolved in binding buffer (0.01 M Tris-HCl, pH 7.5, 0.5 M LiCl, 0.5% SDS, and 1 mM EDTA) and applied to an oligo(dT)-cellulose (type 3, Collaborative Research) column. After washing with binding buffer, poly(A)-containing RNA was eluted with elution buffer (0.01 M Tris-HCl, pH 7.5, 0.05% SDS, and 1 mM EDTA), and RNA was precipitated with ethanol in the presence of 0.1 M NaCl.

**Agarose gel electrophoresis.** Analytical gel electrophoresis was conducted following denaturation of RNA with glyoxal treatment, as described previously (McMaster and Carmicheal, 1977). Preparative gel electrophoresis in 1% urea-agarose gels was performed as described previously (Makino et al., 1984). Since individual intracellular viral RNA species excised from the gels were occasionally contaminated with smaller-size RNAs, the gel slices were melted at 75°C and applied to a second urea-agarose gel. After the second electrophoresis, contaminating RNAs were no longer detected. The RNA was eluted from gel slices by the methods of Langridge et al. (1980).

**Oligonucleotide fingerprinting.** $^{32}$P-labeled and purified viral RNA was digested with ribonuclease T$_1$ and the products were analyzed by two-dimensional polyacrylamide gel electrophoresis as described previously (Makino et al., 1984).

**Fragmentation of virion RNA for oligonucleotide mapping.** The $^{32}$P-labeled purified virion RNA was dissolved in a buffer containing 0.01 M Tris-HCl (pH 7.5) and 1 mM EDTA, and divided into four aliquots. Sodium carbonate was added to each at a final concentration of 0.05 M and then each solution was incubated at 25°C for 1 min or 3 min or at 50°C for 1 min or 3 min. Then the solutions were neutralized with acetic acid and the RNA was precipitated with ethanol in the presence of 0.1 M NaCl. These alkali-degraded RNA fragments were pooled and applied to a urea-agarose gel. After electrophoresis, the gel areas containing RNA fragments of different sizes were excised and RNA was eluted, precipitated by ethanol, selected by oligo(dT)-cellulose column chromatography, and each sized poly(A)-containing RNA was analyzed by oligonucleotide fingerprinting.

**RESULTS**

**Oligonucleotide fingerprinting of genomic RNA of MHV-JHM, 1a, and 2c.** The genomic RNAs of MHV-JHM, 1a, and 2c were analyzed by oligonucleotide fingerprinting and the results are shown in Figs. 1A, B, and C. Prior to RNase T$_1$ digestion the RNAs were analyzed on glyoxal agarose gels (data not shown) and found to have identical electrophoretic mobilities, corresponding to an estimated molecular weight of $5.4 \times 10^6$ (Lai and Stohlman, 1978; Makino et al., 1984). In order to positively identify each oligonucleotide, mixtures of the genomic RNA of MHV-JHM and 1a and also MHV-JHM and 2c were digested together and the resulting oligonucleotide maps are shown in Figs. 1D and E, respectively. Careful examination of these fingerprints shows that more than 45 characteristic oligonucleotides can be identified in the genomic RNA of MHV-JHM. A single oligonucleotide (indicated by an arrow in Fig. 1A) was not reproducibly found. An oligonucleotide fingerprint of this complexity corresponds well to a single-stranded RNA of this size which does not contain extensive sequence reiteration. The oligonucleotide fingerprint of MHV-JHM shown here is also very similar to that described by Stohlman et al. (1982). We have therefore adapted the numbering system used by Stohlman et al., additionally numbering four oligonucleotides which are unique to our MHV-JHM isolate as 2a, 2b, 26b, and 37a.
When the oligonucleotide fingerprint of the wild-type MHV-JHM is compared to those of the mutant viruses 1a and 2c, a number of significant differences can be found and these differences are summarized schematically in Fig. 2. Essentially both mutants lacked the oligonucleotides 2b, 4, 6, 17, and 26b (indicated by arrow heads in Fig. 1A) of the wild-type MHV-JHM, and contained one new oligonucleotide, 26c (arrows in Figs. 1B and C). The mutant 2c also contained three additional oligonucleotides 6c, 48, and 49 (arrows in Fig. 1C).

**Location of the RNase T₁-resistant oligonucleotides on the genome of MHV-JHM.** The genomic RNA of MHV-JHM was degraded by mild alkali treatment and fragments were then fractionated into five size classes by electrophoresis in urea-agarose gels. The 3' cterminal fragments were selected by oligo(dT)-cellulose column chromatography and oligonucleotide fingerprints of each class were prepared. The results are shown in Fig. 3. If the genome of MHV-JHM is assumed to be 18 kilobases (kb) in length (Lai et al., 1981; Siddell, 1983) it can be deduced that the oligonucleotides 4, 17, and 6 and the oligonucleotides 2b and 26b (i.e., those absent from the mutant viruses) were located respectively, between 5.5 to 8 kb, 8 to 10.6 kb, 10.6 to 14.8 kb, and 16.5 to 18 kb from the 3' end of the genome. It should also be noted that the localization of oligonucleotides presented here does

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**Fig. 1.** Oligonucleotide fingerprints of the genomic RNAs of MHV-JHM (A), 1a (B), and 2c (C), a mixture of JHM and 1a (D) and a mixture of JHM and 2c (E). ³²P-labeled purified genomic RNA or equal amounts of ³²P-labeled purified genomic RNAs of JHM and 1a or JHM and 2c were mixed and digested with RNase T₁ and analyzed by two-dimensional gel electrophoresis. (A) Arrow heads indicate the oligonucleotide spots detected only in MHV-JHM. (B, C) The arrows indicate the oligonucleotide spots found in mutant viruses.
not differ significantly from that previously reported (Stohlman et al., 1982) with the exception of oligonucleotide 8 (see also below), which in our study is located near the 5' end of the genome, in contrast to Stohlman et al. (1982) who located this oligonucleotide 5 to 7 kb from the 3' terminus.

Oligonucleotide fingerprinting of intracellular viral RNAs of MHV-JHM, 1a, and 2c. In agreement with previous reports (Lai et al., 1981, 1982; Leibowitz et al., 1981; Spaan et al., 1981; Wege et al., 1981) seven major poly(A)-containing viral RNA species (numbered RNA1 to RNA7 in order of decreasing size) were found in MHV-JHM infected cells (Fig. 4a). These RNAs had estimated molecular weights of 5.4, 3.3, 2.6, 1.25, 1.12, 0.86, and 0.6 × 10⁶. No discrete RNA bands were identified in mock-infected extracts (data not shown). A number of minor virus-specific RNA species were also found in cells infected with both wild-type and mutant viruses.
FIG. 3. Oligonucleotide fingerprints of partially degraded poly(A)-containing MHV-JHM genomic RNA species. The $^{32}$P-labeled purified genomic RNA of MHV-JHM was mildly digested, separated by urea-agarose gel electrophoresis, and poly(A)-containing RNAs were selected by oligo(dT)-cellulose column chromatography. Each sized poly(A)-containing RNA was digested with RNase T$_1$ and oligonucleotides were separated by two-dimensional polyacrylamide gel electrophoresis. The sizes of poly(A)-containing fragmented RNAs used for each oligonucleotide fingerprinting are indicated as 5.5, 8.0, 10.6, 14.8, 16.5, and 18.0 kb. (labeled a, b, c, d, and e in Fig. 4) and the relative proportions of different RNA species was also variable. For example, RNA2 was missing in mutants 1a and 2c infected cells, RNAs 5 and c were particularly prominent in mutant 2c infected cells and RNAa was only irreproducibly found. Oligonucleotide maps of the intracellular RNA species of all these viruses were prepared. In some cases it was difficult to separate major from minor RNA species (e.g., RNA5/c) but possible mixtures are clearly indicated in the figures.

The oligonucleotide fingerprints of intracellular viral RNAs of JHM are presented in Fig. 5. As reported by Lai et al. (1981) the pattern of RNA1 was identical to genomic RNA and the fingerprinting pattern was more complex with increased sizes of RNAs. Most oligonucleotides of the small RNAs were included in neighboring larger RNAs and the localization of oligonucleotides of the intracellular viral RNAs was well matched with the order of oligonucleotides determined in genomic RNA. However, major exceptions were found for oligonucleotide 8, which has been located close to the 5' end of the genomic RNA but appeared in every intracellular viral RNA, and several oligonucleotides (indicated by arrow heads in Fig. 5) which are present in subgenomic RNAs (with the exception of RNA2) but were not found in genomic RNA (see
FIG. 4. Agarose gel electrophoresis of the virus-specific intracellular RNAs of MHV-JHM (a), 1a (b), and 2c (c). Poly(A)-containing \( ^{32}P \)-labeled intracellular viral RNA was isolated by oligo(dT)-cellulose chromatography, denatured with glyoxal and DMSO, and electrophoresed on 1% agarose gel. An arrow indicates the top of the lanes.

Discussion). The oligonucleotide 34 which was found only in RNA1 was numbered 34*.

As the intracellular RNA species of MHV-JHM form a 3' coterminous nested set (Leibowitz et al., 1981), careful analysis of these fingerprints allows for a more detailed localization of the oligonucleotides on the genome, as is shown in Fig. 6. Thus oligonucleotides 17 and 6 could be more accurately positioned between 8 and 8.7 kb and 11 and 14.8 kb, respectively from the 3' end of the genome.

The oligonucleotide fingerprints of the intracellular viral RNA of 1a and 2c are shown in Figs. 7 and 8. The structures of the RNAs were in general agreement with that of MHV-JHM. Oligonucleotide 8 is again present in each subgenomic RNA and subgenomic RNAs contain oligonucleotides which are not found in genomic RNA. Most surprisingly oligonucleotide 2, which in MHV-JHM infected cells was found only in RNA6 and larger RNAs, was conspicuously present in RNA7 of mutant 2c (see Discussion).

Using these fingerprints it was also possible to locate the position of the oligonucleotides which was specific to the mutant viruses. Oligonucleotide 26c (indicated in both mutants but not in MHV-JHM) was located 5.5 to 8.7 kb from the 3' end of the genome. In the mutant 2c, the oligonucleotides 6c, 48, and 49 are located 8.7 to 18 kb, 3.8 to 4.1 kb, and 8.7 to 18 kb from the 3' end of the genome, respectively. A number of oligonucleotides appeared to give locations in the mutant intracellular RNAs which were inconsistent with their locations in MHV-JHM intracellular RNA. These oligonucleotides are designated 48*, 11*, and 34* (for example, oligonucleotide 48 is found in RNAs 1 to 5 of MHV-JHM, but only in RNA1 of the mutant viruses). Oligonucleotide 35 was found in RNA1 to RNA5 of MHV-JHM, but only in RNA1 to 4 of mutant viruses. Although we are sure these anomalies are significant we cannot readily explain their occurrence.

Oligonucleotide fingerprinting of genomic RNA of viruses released from different passage levels of JHM-CC. The mutant viruses 1a and 2c were isolated from the JHM-CC culture at the 133rd passage. In order to search for any genotypic changes in the RNA of viruses released from the JHM-CC culture over a period of time, we infected DBT cells with JHM-CC virus from different passage levels, labeled with \( ^{32}P \)orthophosphate, purified the progeny virus, and extracted and analyzed the viral RNA by oligonucleotide fingerprinting. At no time did we detect any alteration in the size of the genomic RNA (data not shown) and the fingerprints of passage 93 virus (93V), passage 110 virus (110V), passage 133 virus (133V), and passage 185 virus (185V) are shown in Fig. 9. These fingerprints show that the genomic RNA of the virus populations 93V, 110V, and 133V were identical to that of the isolated
FIG. 5. Oligonucleotide fingerprints of the intracellular MHV-JHM-specific RNAs. The virus-specific RNAs were eluted from urea-agarose gels, digested with RNase T₁, and oligonucleotides were separated by two-dimensional polyacrylamide gel electrophoresis.

mutant 1a. The genomic RNA of 185V has one additional oligonucleotide (48) which is also found in the isolated mutant 2c.

DISCUSSION

The results presented here confirm and extend previous studies on MHV, strain JHM, and the related MHV strain A59 (Lai et al., 1981, 1982; Leibowitz et al., 1981; Spaan et al., 1981). The detailed location of a large number of RNase T₁-resistant oligonucleotides on the genomic RNA and oligonucleotide mapping of the seven intracellular RNA species provide
The proposed seven genetic regions are based on the data described in the present study and from others (Lai et al., 1981; Siddell, 1983). Each region corresponds to the position of each viral intracellular RNA species which does not overlap with the neighboring-intracellular RNA species.

These experiments also identify for the first time oligonucleotide 8 as being derived from sequences which are encompassed within the leader of MHV-JHM. Also in each subgenomic RNA of MHV-JHM (with the exception of RNAZ) oligonucleotides can be identified which are not found in genomic RNA and it can be assumed that these “unique” oligonucleotides have arisen from the combination of sequences during the synthesis of mRNAs involving the joining of leader and body transcripts (Lai et al., 1983b; Spaan et al., 1983). It is interesting to note that at least for some of the subgenomic RNAs (e.g., RNA7 and RNA6) these “unique” oligonucleotide are apparently very similar in their electrophoretic behavior, suggesting that the sequences combined during RNA synthesis may be very similar. In other cases, however, e.g., RNA5, the “unique” oligonucleotide is clearly different, suggesting the combination of different sequences (the differences most probably being derived from the mRNA body). In one case, RNAb, two such oligonucleotides are identified. The explanation of this result requires further study but it is perhaps noteworthy that in the case of mutant virus RNAs (see below) such a result is found when a mixture of two RNAs are analyzed.

Oligonucleotide analysis of the mutant virus genomic RNA and subgenomic RNAs leads to two conclusions. First, regarding the synthesis of subgenomic RNAs; in RNA7 of mutant 2c one oligonucleotide, 2, is clearly found which has been mapped.
in wild-type MHV-JHM to the sequences unique to RNA6 and large RNAs. The simplest explanation of this result would be that in the mutant virus the body of RNA7 extends further in a 5' direction and includes sequences not found in the body of wild-type MHV-JHM RNA7. One possible interpretation would be therefore that during mRNA synthesis alternative combinations of leader and body sequences may be joined. It is also important to note that in mutant 2c the “unique” oligonucleotide in RNA7 appears to be the same as in wild-type MHV-JHM subgenomic RNA, but is clearly different in the case of RNA6. These conclusions should however be considered tentative, because alternative more complex explanations involving rearrangements of sequences in the genomic RNA are conceivable. Even-
Fig. 8. Oligonucleotide fingerprints of the intracellular 2c-specific RNAs.

Eventually sequence analysis of these RNA species and the corresponding regions of the genomic RNA will be necessary to provide a full explanation.

The second conclusion that can be reached by comparison of the wild-type MHV-JHM and mutant viruses concerns the locations of detectable sequence changes in the viruses which replicate in the persistent culture JHM-CC. In Fig. 6 it can be seen that with the exception of the sequences surrounding oligonucleotide 48, all of the sequence changes in the mutant viruses are located (or in the case of oligonucleotides 6c, 49, and 43*, may be located) in one of two regions of the genome, namely in the unique sequences found in genomic RNA (or RNA1) and
FIG. 9. Oligonucleotide fingerprints of genomic RNA of viruses released from JHM-CC at different passage levels. The viruses isolated from JHM-CC at passage levels 93, 110, 133, and 185, respectively are indicated as 93V, 110V, 133V and 185V.

the sequences between the 5' termini of RNAs b and 3. These regions of the genome are believed to encode the RNA polymerase functions (RNA1) (Brayton et al., 1982; Mahy et al., 1983) and the E2 peplomer protein of the virion (RNA3) which is responsible for virus adsorption into cells (Holmes et al., 1981; Niemann and Klenk, 1981; Rottier et al., 1981a). The fact that in tissue culture both mutants grew more slowly than wild-type MHV-JHM, and possess altered E2 proteins (Makino et al., 1983) may, but need not necessarily, be related to these changes.
In the infected animal it has been suggested that MHV-JHM induced demyelination is a consequence of the lytic infection of oligodendrocytes (Knobler et al., 1981) and the observation that both mutants produced reduced neural degeneration and delayed demyelination (unpublished observation) might be related to the inability of the viruses to infect neural cells, together with reduced rates of replication in oligodendrocytes.

It is now recognized that most, if not all, RNA viruses have mutation frequencies, in part because of the lack of proof-reading enzymes that assure fidelity of DNA replication. The high rates of replication of RNA viruses also contribute to this high mutation frequency (Holland et al., 1982). Oligonucleotide fingerprinting of the virus populations released from different passages of the JHM-CC culture and isolates from the culture provides evidence that mutations are readily produced and accumulated in the MHV-JHM genome. This conclusion would also support previous descriptions of MHV gene diversification reported by Lai et al. (1983a). The oligonucleotide fingerprintings also show that the virus population released from the JHM-CC culture is relatively constant, although the differences shown for the mutants 1a and 2c, which were both isolated from the 133V population, demonstrate that individual viruses within the population can have different genetic structures. In the JHM-CC culture there does not appear to be any fluctuating emergence of variants which predominate the culture at any one time. This is in contrast to the situation reported in several other virus systems (Holland et al., 1979, 1982; Meinkoth and Kennedy, 1980), although in these cases defective interfering (DI) particles appeared to play a role, while in the JHM-CC culture there is no evidence for the presence of DI particles (Hirano et al., 1981).

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