Chromatography of Mouse Hepatitis Virus Replicative Intermediate and Replicative Form RNA

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1. INTRODUCTION

One of our goals is to obtain large amounts of selected size classes of the coronavirus replicative intermediates (RI) and native replicative form (RF) RNA that contain the templates for viral genome and messenger RNA syntheses. The two replicative molecules differ in their relative amounts of single-stranded and double-stranded character, with the RF RNA considered essentially or completely double-stranded. It is known that there are seven species of RI RNA, each of which is proportional in size and abundance to one of the seven species of viral messenger RNA produced in infected cells (Sawicki and Sawicki 1990; 1998; Schaad and Baric 1994; Baric and Yount 2000). The presence in coronavirus-infected cells of seven viral minus strand templates was first reported by Sethna and Brian (1989). To assist in this effort, we undertook an analysis of how mouse hepatitis virus (MHV-A59) RI and RF RNA fractionate on three gel filtration matrices, Sepharose 2B, Superose 6 and Sephacryl S-1000. A second goal was to attempt to explain the failure by others to find subgenomic RI and their RF cores after ribonuclease treatment and gel filtration chromatography.

2. RESULTS

17Cl-1 cells were infected with MHV-A59 at an MOI of 65-80 and continuously labeled in the presence of 20 μg of actinomycin D/ml with ^{32}P -orthophosphate in phosphate-reduced medium (containing 1% normal levels) from 1.5-5 h p.i. The cells were harvested into buffer containing 5% lithium dodecyl sulfate and 200 μg of proteinase K/ml. The RNA was deproteinized with acid phenol and chloroform, and collected by ethanol precipitation. The total infected cell RNA was applied to a 1.5 x 90 cm column of Sepharose 2B (Pharmacia), as described in earlier studies (Sawicki and Gomatos 1976). Viral RNA was both excluded from the column, indicating these were equal to or larger than the exclusion limit of 1.3 kilobase-pairs of DNA or about 1×10^6 Daltons, and included or within the fractionation range of the column (Fig 1). RNA in each fraction was collected by ethanol precipitation, digested with 10-30 units of ribonuclease T1/sample and analyzed on 1% agarose/TBE gels. Seven species of double-stranded RI cores or RF were found (Fig 1). The results indicate the largest three RIs are excluded by this matrix and the four smallest fractionate by size as they elute in order. However, they elute broadly, over a large number of fractions, and different RI species elute together (overlap).

In a second approach, we combined velocity sedimentation and chromatography. Total infected cell RNA was applied to a 15 to 30% sucrose gradient and sedimented to pellet single-stranded RNA 1 and place 28S ribosomal RNA in fraction 6 of 30 fractions and 18S ribosomal RNA in fraction 15 of 30, the middle of the gradient. RNA in each fraction was collected, digested with ribonuclease T1 and then electrophoresed on agarose gels. The results indicate velocity sedimentation separated genomic RF 1 from smaller RF cores, which sedimented according to size but again overlapped extensively (Sawicki, manuscript in preparation). These were collected into 4 pools, numbered starting from the bottom of the gradient, and pools 2 and 4 were individually chromatographed on Sephacryl S-1000 columns (data not shown). Sephacryl S-1000 is a hydrophobic, rigid, allyl dextran/N-N'-methylenebisacrylamide matrix (Pharmacia) with a 20 kilobase-pair exclusion limit. To summarize the results, pool 2 RNA (~40S to 30S) was totally excluded by the column and contained the three largest RI cores, RF I-III. Early fractions contained only RF I; later fractions were enriched in RF II and RF III. Pool 4 RNA (16S to 10S) gave 2 peaks: a minor, excluded one containing small amounts of the three largest RF cores and a major, included peak containing the four smallest cores, RF IV-VII. Sephacryl S-1000 gave a better profile for the four smallest RF molecules, which eluted very narrowly and by size, than Sepharose 2B. But, even on this matrix, elution of each RF overlapped with that of at least one other.

Figure 1. Chromatography on Sepharose 2B of MHV-infected cell RNA labeled with ^{32}P -orthophosphate from 1.5-5 h p.i.. RNA larger than 3 million Daltons eluded in fractions 47-60 (excluded region). RNA in fractions 47-87 was analyzed by gel electrophoresis.

The elution profile of these molecules by FPLC on a Superose 6 column, a cross-linked agarose matrix similar to Sepharose 2B but having a particle size of 10-13 microns versus 60-200 microns for Sepharose 2B, resembled that of Sepharose 2B (Fig 2). The shorter column length, higher pressure during FPLC chromatography and/or smaller particle size may contribute to the observed retention of the three largest RF, especially RF I, and its elution even in fractions beyond those containing RF VII molecules that are 5% the size of RF I. Only Sephacryl S-1000 of the three matrices tested was

able to include and fractionate all subgenomic RF. Of special note, comparison of the gel filtration results indicated coronavirus RI and RF RNA chromatographed close to what was predicted from the fractionation properties of each matrix.

2.1 There are Seven Native RI and Seven Native RF RNA

We next obtained native RF RNA. Native RF are soluble in 2M LiCl from their having no or very little single-stranded character. In contrast, native RIs have extensive single-stranded character and precipitate in high salt, as do single-stranded mRNA and ribosomal RNA. Infected cells were incubated continuously in medium containing 200 μ Ci of 3 H-uridine/ml and actinomycin D from 1.5-5.5 h p.i. to obtain RF and RI RNA labeled in both their plus and minus strand components. After deproteinization with acid-phenol, the total infected cell RNA was adjusted to 2M with LiCl, placed on ice for 18 hours, and centrifuged at 10,000 rpm for 1 hour to obtain the supernatant (LiCl soluble fraction) and precipitated (LiCl insoluble) fractions. The LiCl soluble RNA was collected by ethanol precipitation and directly chromatographed on Sepharose 2B. To summarize, three peaks of labeled RNA were found, the largest of which was excluded. Fractions were pooled and each pool was analyzed on gels without nuclease treatment. Pool 1 was the excluded peak, and pools 3 and 4 were the leading and trailing regions of the included first peak, respectively. Pool 1 was found to contain all of the largest three RF classes and small amounts of RF IV-VI. Pool 3 was enriched in the four smallest RF classes, and pool 4 contained predominantly RF VII. Thus, infected cells contain seven species of native RF RNA.

Figure 2. FPLC Chromatography of MHV-A59 RF RNA on Superose 6 gel filtration.

The LiCl insoluble fraction was also chromatographed on Sepharose 2B. Its amount of excluded RNA was >10 times greater than in the LiCl soluble fraction. The included RNA peak displayed a large and heterogeneous range of sizes, as expected for the seven species of viral mRNAs. RNA in alternate fractions was analysed on agarose gels directly to identify the elution position of each viral RNA species (fractions of 0.65, half the size of the LiCl soluble analysis, were collected for the LiCl insoluble RNA to attempt to obtain better resolution). Ethidium bromide staining of the gel indicated where 28S and 18S ribosomal RNA eluted. The results showed that genome or single-stranded RNA 1 eluted in the excluded volume as did the largest native RI, which was visible at the top of the gel in fractions 60-65. It was especially noted that RI 1 migrates slower than its single-stranded product on agarose gels, as do all of the RI or RF RNA relative to their single-stranded counterparts (Sawicki and Sawicki 1990). Eluting in an overlapping pattern, according to their relative sizes, and after the genome RNA were single-stranded mRNA 2-7. Viral mRNA 2-5 overlapped the elution of 28S ribosomal RNA, and mRNA 4-7 overlapped the 18S ribosomal RNA (Sawicki, in preparation).

About every 5 fractions of the Sepharose 2B fractionated LiCl insoluble RNA were pooled (numbered 1-20 beginning with the excluded fractions), treated with ribonuclease T1 and analyzed on gels to identify the elution profile of individual native RI classes. Pool 1 RNA contained most of the three largest RI classes and some single-stranded RNA, accounting for its high (49%) resistance to digestion when assayed for acid-precipitability after digestion with 5 µg of pancreatic ribonuclease/ml in 0.3 M NaCl or 2 x SSC. Pools 6-7 were enriched in the smallest, RI VII, and the other RI eluted in pools 2-5. We found the seven, native RI classes separated similarly to native RF RNA on Sepharose 2B and each eluted ahead of its single-stranded RNA counterpart, as expected.

2.2 Ribonuclease-resistance of Native RF and RI RNA

We also determined that native RF were fully double-stranded and that native RI were partially double-stranded. Digestion with 10-100 units of ribonuclease T1 left all seven RF species intact as judged by their identical migration on gels to untreated RF species. And, essentially 100% of labeled, native RF RNA was acid-precipitable after digestion with either ribonuclease T1 (30 units/sample) in 0.3 M NaCl at 30°C for 30 min or ribonuclease A (5 µg/ml) in 0.3 M NaCl or 2xSSC at 37°C for 30 min. While ribonuclease T1 treatment left native RF intact or generated RF cores from native RI RNA, overdigestion with ribonuclease A degraded coronavirus RI and RF molecules (Sawicki, in preparation). Ribonuclease in

excess of 1 µg/ml destroyed large and midsize RF or RI RNA; higher amounts degraded small RF or RI species.

3. CONCLUSIONS

Seven RF and RI molecules are readily detected after velocity sedimentation or after chromatography. Sepharose 2B excluded RI I-III and fractionated the others by size; Sephacryl S-1000 excluded mainly RI/RF I and gave the best fractionation of all subgenomic RI and RF RNA. However, even Sephacryl S-1000 did not eliminate the overlap of the two mid-sized molecules with each other, and of the four smallest RI/RF molecules with each other. Thus, electrophoresis on gels remains the best method to purify individual size-classes of native RF or RI RNA (Sawicki and Sawicki, 1990). Combining sedimentation and gel filtration provided enriched populations of large (I or I-III) size-classes or small (IV-VII) size-classes of RF and RI RNA.

The initial failure to find subgenomic RI RNA led to the leader-primed transcription model (Baric *et al* 1983). Our results indicate this failure was likely due to technical error and not to any aberrant behavior of these molecules on gel filtration chromatography. Concentrations of ribonuclease A used by others of 10 µg/ml (Baric *et al* 1983) and 20 µg/ml (Lai *et al* 1982) destroyed viral RI and native RF molecules. Moreover, RF I migrates on gels slower than genome RNA 1, not faster as reported (Baric *et al* 1983), indicating that genome RF I was not recovered under conditions used by Lai and coworkers in 1982 and 1983. Evidence arguing against the leader-primed transcription model is now extensive and includes work by our lab and that of Eric Snijder and Willy Spaan (van Marle *et al* 1999), of David Brian (Sethna *et al* 1989) and of Ralph Baric (Baric and Yount 2000); and this proceedings). This evidence and the ability to explain the initial failure to find subgenomic RI and RF RNA lead us to state that the leader-primed model has been disproved.

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