ANALYSIS OF MESSENGER RNA WITHIN VIRIONS OF IBV

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ABSTRACT

The presence of subgenomic mRNAs (sgRNAs) in virions of infectious bronchitis virus was examined by probing Northern blots of RNA extracted from virions using as a probe a cDNA of the 3'-terminal nucleocapsid protein (N) gene. The sgRNAs were readily detected even after extensive purification of virions and after RNase A treatment of virions. The molar ratio of gRNA to each sgRNA was in the range 25 to 400 for IBV-M41 and 10 to 30 for IBV-Beaudette. After comparison with the molar ratios of genomic to intracellular viral sgRNAs it was estimated that the efficiency of incorporation of gRNA into virions was approximately 100 to 500-fold greater than for sgRNAs in the case of M41 and 20 to 100-fold for Beaudette, depending on the sgRNA species. It is concluded that sgRNAs can be present within IBV virions. Approximately 1 in 3 Beaudette virions and 1 in 20 M41 particles might contain a single copy of one sgRNA.

INTRODUCTION

Recently sgRNAs of transmissible gastroenteritis virus (TGEV) and bovine coronavirus (BCV) have been reported to be present in virions[1-3] although no very small defective-interfering (DI) RNAs were packaged into particles of murine hepatitis virus (MHV).[4] We have investigated the presence of sgRNAs in virions of IBV.

METHODS

Virus growth and RNA extraction

Most work was performed with M41 and IBV-Beaudette which were grown in Vero cells (Beaudette only), chick kidney (CK) cells and embryonated fowl eggs. IBV was

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radiolabelled with ^{32}P-inorganic phosphate in CK cells and cell-associated RNA (CK cells and chorioallantoic membrane, CAM, from infected embryonated eggs) and RNA in pelleted virions was extracted using guanidinium isothiocyanate[5]. RNAs were separated in 1.2% agarose gels containing formaldehyde and the gel exposed to u.v. light (302 nm) for 2 min to nick the RNA sufficiently to improve the transfer of gRNA to nitrocellulose filters. The filters were probed with a cDNA of the N gene produced by the polymerase chain reaction (PCR). Radiolabelled (^{32}P) probes were made by the random hexanucleotide primer method and non-radioactive probe was made and used in accordance with the manufacturer's instructions for the ECL (enhanced chemiluminescence) direct nucleic acid labelling and detection system (Amersham International). The RNAs in extracts of ^{32}P-labelled infected cells were separated in agarose gels as described above. The gel was dried, an autoradiograph prepared and superimposed on the gel, bands were excised and the radioactivity determined in a scintillation counter in order to calculate the gRNA/sgRNA molar ratios of cell-associated viral RNA.

Differential purification of IBV virions

Virus was purified at 0-4°C and RNA extracted from some virions at each stage. Briefly, allantoic fluid from infected eggs was clarified, and then the virions centrifuged to produce primary pellets of virions. The resuspended virions were then pelleted through 25% (w/w) sucrose onto a 55% sucrose pad to give banded virus. This was then sedimented under isopycnic conditions through a linear 25-55% sucrose gradient.
After fractionation of the gradient and measurement of the A_{260} of each fraction, fractions 13 to 25 were pooled in pairs, diluted, and the virions pelleted (isopycnic gradient purified virus).

RNase A treatment of IBV virions

Briefly allantoic fluid from eggs infected with IBV-M41 was harvested, clarified and the virus pelleted, resuspended in NET buffer (100 mM NaCl, 1 mM EDTA, 10 mM tris-HCl, pH 7.4) and divided into 90 ul aliquots. Some aliquots were then incubated at 37°C for 5 or 30 min with RNase A (Sigma). Virions in other samples were first treated with 2% Nonidet P40 non-ionic detergent (BDH) to dissolve the virus envelope before addition of RNase. Controls were non-treated virus simply incubated at 37°C for 5 and 30 min and virus incubated with NP-40 after addition of RNAguard RNase inhibitor (Pharmacia). The RNA was extracted by addition of 2 ml of solution D,[5] 5 ug of tRNA added and the RNA purified. Half of each sample, equivalent to the virus from about 2.5 eggs, was used for electrophoresis.

RESULTS

Presence of IBV sgRNAs in preparations of virions

In addition to the gRNA and 5 sgRNAs (corresponding to mRNAs) previously described for IBV-Beaudette our analysis of many strain of IBV frequently revealed the presence of two additional sgRNAs, indicated by arrows in Figs. 1 and 2. The band between sgRNAs 2(encoding the spike,S, glycoprotein) and 3 hybridised to a cDNA probe corresponding to the 3' half of the S gene but not to any of six probes corresponding to

regions throughout the polymerase (pol) gene, including the first 2 kb at the 5' terminus. The other band, between sgRNAs 4(M) and 5 did not bind any of the pol and S gene probes (data not shown).

RNA extracted from primary pellets of virions of Beaudette included sgRNAs (Fig.1, lane e). Comparison with that extracted from infected Vero and CK cells (Fig. 1, lanes b and d) clearly showed that the gRNA/sgRNA ratios were greater for virions than for intracellular RNA, indicating that the sgRNAs had been incorporated at lower efficiency than gRNA. When Northern blots were probed with N gene-specific positive- and negative-sense radiolabelled oligonucleotides, only binding of the negative-sense oligonucleotide was detected, showing that the great majority of the molecules were plus-sense (mRNAs). The ratios for gRNA/sgRNA did not change with increased purification of virions (Fig. 2). Lanes (c) to (h) in Fig. 2 also show that after sucrose gradient sedimentation of M41 virions the fractions containing most sgRNA coincided with those containing most gRNA, which in turn corresponded to the peak fraction of virions as determined by A_{260} readings (not shown). This showed a close association of the sgRNAs with the virions.

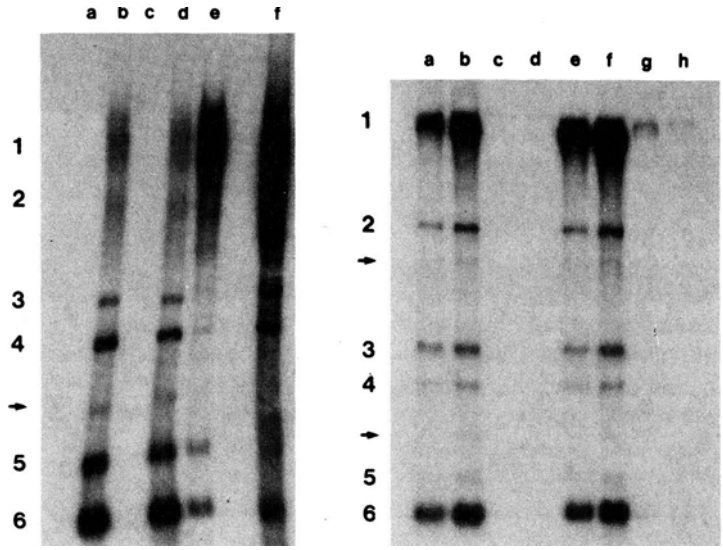

Figure 1. Northern blot of IBV RNAs probed with a ^{32}P-labelled N gene probe: RNA from mock-infected (a) Vero and (c) CK cells; cell-associated RNA from infected (b) Vero and (d) CK cells; virion-associated RNA from IBV strains (e) Beaudette and (f) UK/142/86. The arrow indicates an IBV RNA species which has not been identified as a functional mRNA.

Figure 2. Northern blot, probed with a ^{32}P-labelled N gene probe, showing the continued presence of sgRNAs during purification of M41 virions: (a) primary pelleted virions; (b) banded virions; (c) to (h) isopycnic sucrose density gradient fractions in the region of the virus peak. The arrow heads indicate two IBV RNA species which have not been identified as functional mRNAs.

Virion-associated sgRNAs were not susceptible to RNAase A

To determine if the sgRNAs were within virus particles, virions were incubated with RNase A, on the premise that the sgRNA would be digested if it were external to the virions. Fig. 3 shows that no viral RNA was destroyed at the highest concentration (10

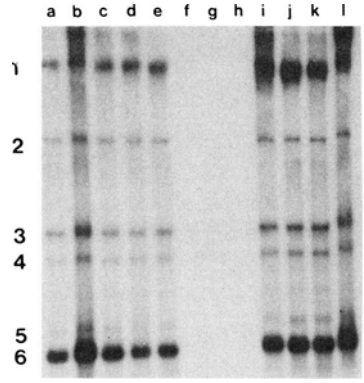

Figure 3. Northern blot, probed with a ^{32}P-labelled N gene probe, showing resistance of virion-associated sgRNAs to RNAase A. RNA was extracted from M41 virions after incubation at 37°C under the following conditions: (a) and (l) without either detergent Nonidet P-40 or RNAase; (b) with Np-40 but without RNAase; (c) to (e) five min incubation without NP-40 but with RNAase at (c) 0.1, (d) 1 and (e) 10 ug/ml; (f) to (h) as for (c) to (e), respectively, but with NP-40 to expose the RNAs previously within the virions; (i) to (k) as for (c) to (e), respectively, but longer (30 min) incubation.

ug/ml, 30 min, 37°C) of Rnase used whereas all RNA was destroyed when the virion membrane was dissociated with non-ionic detergent.

Low efficiency of incorporation of sgRNAs into virions

The molar ratios of the RNAs in virions were estimated from the amount of N gene probe bound to Northern blots. The 3' co-terminal nested set nature of the RNAs means that each molecule of RNA, irrespective of size, should bind the same amount of probe and hence the amount of probe bound is in proportion to the molar ratio of the RNAs. Account was taken of the finding that transfer of gRNA from agarose to nitrocellulose was only 63% efficient (mean of three separate transfers of radiolabelled IBV RNA) compared with almost 100% for the sgRNAs. The molar ratio of M41 RNAs was estimated from blots of dilutions of virion RNA probed with an ECL probe (data not shown). Comparison of band intensities indicated that the molar ratio gRNA/sgRNA ranged from about 25 for sgRNA 6 to 400 for mRNA 5. When account was taken of the molar ratios of gRNA/sgRNA within cells (not shown) i.e. in which most sgRNAs outnumber gRNA, the efficiency of incorporation of gRNA was estimated to be 100 to 500-fold greater for sgRNAs. The molar ratio for virion RNAs of Beaudette was calculated from blots probed with a ^{32}P-labelled probe, the gRNA/sgRNA ratio ranging from about 10 for sgRNA 6 to 30 for sgRNAs 3 and 4. The efficiency of incorporation of gRNA was 20 to 100-fold greater than for sgRNAs.

DISCUSSION

The presence of sgRNAs in virions is relevant to several aspects of coronavirus replication, including the requirement of signals for packaging of RNA into virions,[7,8] the capacity of coronavirus sgRNAs to function as replicons[1-3,9] and, following the experimental demonstration of recombination during MHV replication[10] and circumstantial evidence for recombination in the field for IBV,[11-13] the possibility that incorporation into

virions of sgRNAs from both parents following mixed infection could increase the chance of recombination at subsequent replication cycles.

Estimates of the molar amounts of sgRNAs incorporated into coronavirus virions vary. Only minute amounts of very small DI RNAs were incorporated into MHV particles.[4] Sethna *et al.*[3] have reported that virions of TGEV contained 5 to 14-fold more gRNA than given sgRNAs, the N protein-encoding sgRNA being the most abundant. Bovine coronavirus (BCV) virions have been reported to contain more molecules of N and M protein-encoding sgRNAs than gRNA.[2] Our results for IBV fall somewhere in the middle. The amounts of sgRNAs in virions of IBV-Beaudette were less than but similar to those reported for TGEV, whereas the frequency of sgRNAs in M41 particles was about 4-fold less than in Beaudette. The majority of IBV virions probably do not contain any sgRNA species. The IBV sgRNAs were incorporated into virions broadly in proportion to the amount of each sgRNA in infected cells. An exception, with both Beaudette and M41, was sgRNA 2, encoding the spike protein, which was over represented.

The efficiency of incorporation of sgRNAs into IBV virions was some two orders of magnitude less than that of gRNA. This is not surprising in view of the finding that a sequence near the 3' end of the gene encoding open reading frame 1b of the 5'-most gene, that encoding the polymerase, is essential for packaging of RNA into virions of MHV.[6-8] None of the sgRNAs of IBV contain any part of gene 1. Our results support the view that specific sequences, present within gene 1, are necessary for efficient packaging i.e. for the formation of the ribonucleoprotein i.e. viral RNA surrounded by N protein molecules.

What role, if any, might intra-virion sgRNAs have in replication? It has been demonstrated for TGEV and BCV that during virus replication sgRNAs can function as replicons; infected cells contain negative sense versions of the mRNAs.[1,3,9] It is conceivable, therefore, that sgRNAs within virions might, upon their release from virions following penetration of host cells, be replicated. Indeed, it has been observed that virion-associated sgRNAs appeared to have served as templates for their own replication following infection of cells with BCV.[2] If this were to occur then some coronavirus particles would, in effect, have two copies of some genes which might result in over-production of the corresponding mRNA and, presumably, of the corresponding encoded protein. This could, conceivably, have an effect on replication by disturbing the balance of the various gene products. However, Makino *et al.*[14] have shown that when MHV-infected cells were transfected with sgRNA, the latter was not replicated. Input mRNA might be translated, but the effect of this would be expected to be minor compared with the translation of the ultimately much greater amount of mRNA produced de novo.

Another way in which virion-associated sgRNA might have a biological effect is in respect of recombination. During template-switching the polymerase might continue RNA synthesis on the input sgRNA and then switch back to synthesis on genomic RNA. (Recombination could also occur during production of plus-sense RNA if the input sgRNA were to be first replicated to produce negative-sense sgRNA). This could result in a recombinant if the input sgRNA was not homologous to the input gRNA. That is, during infection of a cell with two strains of virus, some progeny virions might have the gRNA from one parental strain and a sgRNA from the other. Of course, recombination could occur during the initial mixed infection. However, if virion-associated sgRNAs can be replicated i.e. serve as a template for the polymerase, then this extends the possibility of recombinants being produced when such virions infect other cells, including in other host individuals.

In conclusion, plus-sense sgRNAs were incorporated into virions of IBV, however the efficiency of incorporation was low and most particles would lack any sgRNA. Whether virion-associated sgRNAs play any significant role in coronavirus replication and recombination depends on whether input sgRNAs can serve as substrates for the virus polymerase.

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