

Presence of Subgenomic mRNAs in Virions of Coronavirus IBV

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The presence of subgenomic mRNAs in virions of IBV 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. This detects all five mRNAs because of the 3'-coterminal, nested-set arrangement of coronavirus mRNAs. The mRNAs were readily detected even after extensive purification of virions and after RNase A treatment of virions. In sucrose gradients the peaks of virus particles, genomic RNA (gRNA), and mRNAs were coincident. Cellular mRNA was not detected in virions. The molar ratio of gRNA to each mRNA ranged from about 10 to 30 for IBV-Beaudette and 25 to 800 for IBV-M41. The molar ratio of genomic to intracellular viral mRNAs was also determined. From this it was estimated that the efficiency of incorporation of gRNA into virions was at least 20- to 100-fold greater, depending on the mRNA species, for IBV-Beaudette and 100- to 500-fold for IBV-M41. It is concluded that most virions contain only gRNA or contain only one species of mRNA on average in addition to gRNA. © 1993 Academic Press, Inc.

INTRODUCTION

IBV is a member of the genus *coronavirus*, family Coronaviridae, and has a nonsegmented, single-stranded, positive-sense RNA genome of approximately 27.6 kb (Bournsnell *et al.*, 1987). Transcription produces five subgenomic mRNAs which form a 3'-coterminal set of mRNAs ranging in size from 2.1 to 7.3 kb (reviewed by Spaan *et al.*, 1988; and Lai, 1990). The gRNA functions as an mRNA, the 5'-most gene of the gRNA composed of approximately 19 kb and encoding the RNA-dependent RNA polymerase (Bournsnell *et al.*, 1987). Recently mRNAs have been detected within virions of the coronaviruses transmissible gastroenteritis virus (TGEV) and bovine coronavirus (BCV) (Sethna *et al.*, 1989; Hofmann *et al.*, 1990; Sethna *et al.*, 1991) but not in virions of murine hepatitis virus (MHV), although small defective-interfering (DI) RNAs were incorporated at low efficiency in addition to large DI RNA at high efficiency (Makino *et al.*, 1985, 1988). The presence of mRNAs in virions is relevant to several aspects of coronavirus replication, including the requirement of signals for packaging of RNA into virions (van der Most *et al.*, 1991; Makino *et al.*, 1991; Fosmire *et al.*, 1992), the capacity of coronavirus mRNAs to function as replicons (Sethna *et al.*, 1989; Sawicki and Sawicki, 1990; Hofmann *et al.*, 1990; Sethna *et al.*, 1991), and, following the experimental demonstration of recombination during MHV replication (Lai *et al.*, 1985; Makino *et al.*,

1986) and circumstantial evidence for recombination in the field for IBV (Cavanagh and Davis, 1988; Kusters *et al.*, 1989, 1990; Cavanagh *et al.*, 1992), the possibility that incorporation into virions of mRNAs from both parents following mixed infection could increase the chance of recombination at subsequent replication cycles.

MATERIALS AND METHODS

Viruses and cells

Most work was performed with IBV-Beaudette and IBV-M41 which had been adapted to growth in chick kidney (CK) cells and, in the case of Beaudette, subsequently in Vero cells, and the viruses grown in Vero and in CK cells (Stern and Kennedy, 1980) and in the chorioallantoic membrane (CAM) of embryonated fowl eggs (for 20-24 hr). Other strains used included NL/D1466/78 and UK/123/82, after adaptation to CK cells, and UK/142/86.

Preparation of viral RNAs

IBV was radiolabeled with [³²P]inorganic phosphate (Amersham International) essentially as described by Stern and Kennedy (1980). Cell-associated RNA (CK cells and the CAM from infected embryonated eggs) and RNA in pelleted virions was extracted using guanidinium isothiocyanate (Chomczynski and Sacchi, 1987).

Analysis of unlabeled viral RNAs

RNAs were separated in 1.2% agarose gels containing formaldehyde (Sambrook *et al.*, 1989) and the gel

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was exposed to uv light (302 nm) (Ultra Violet Products) 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 IBV N gene, produced by the polymerase chain reaction (PCR; Sambrook *et al.*, 1989) or a cDNA of the mRNA for chicken polypeptide chain elongation factor 1 α (a gift of Dr. N. Bumstead of this Institute). Radiolabeled (^{32}P) probes were made by the random hexanucleotide primer method and nonradioactive probe was made and used in accordance with the manufacturer's instructions for the ECL (enhanced chemiluminescence) direct nucleic acid labeling and detection system (Amersham International). The amount of PCR product used to make the ECL probe was 2 μl from an 80 μl PCR reaction. X-ray film (Hyperfilm-MP, Amersham) was exposed to ECL-probed filters for 5–60 min and to filters exposed to radioactive probes for 1–2 days.

Analysis of labeled viral RNAs

The RNAs in extracts of ^{32}P -labeled infected cells were separated in agarose gels as described above. The gel was dried onto GelBond (FMC BioProducts), an autoradiograph prepared and superimposed on the gel, bands were excised, and the radioactivity determined in a scintillation counter. The relative molar amounts of each IBV RNA species were calculated by dividing the radioactivity in each band by the size, in kilobases, of the respective RNA species. The sizes of the IBV-Beaudette RNA species used were: RNA 1, 27.6 kb; RNA 2(S), 7.3 kb; RNA 3, 3.9 kb; RNA 4(M), 3.3 kb; RNA 5, 2.5 kb; and RNA 6(N), 2.1 kb.

Differential purification of IBV virions for RNA extraction

Virus was purified at 0–4°. Allantoic fluid from infected eggs was clarified (4000 g , 30 min). A proportion (10%) of the clarified fluid was centrifuged at 27,000 g for 4 hr to produce primary pelleted virus from which the RNA was extracted. Virions in the remainder of the fluid were pelleted at 35,000 g for 1.5 hr. The resuspended virions were divided into two portions (approximately 10 and 90%). The smaller amount was underlaid with 25% (w/w) sucrose and the larger amount placed on top of 25% sucrose under which was 55% sucrose. Both samples were centrifuged at 47,000 g_{av} for 2.5 hr. RNA was extracted from the smaller amount (banded virus). The virus at the 25/55% sucrose interface was diluted, placed on top of a 25–55% linear sucrose gradient, and spun at 49,000 g_{av} for 16 hr. After fractionation of the gradient and measurement of the A_{260} of each fraction to semiquantify the virus, fractions 13 to 25 were pooled in pairs, diluted, the virions pelleted at 106,000 g for 1.5 hr, and the RNA was extracted (isopycnic gradient-purified virus).

TABLE 1
RELATIVE MOLARITIES OF IBV-BEAUDETTE AND IBV-M41
INTRACELLULAR mRNAs

mRNA species	Molarity relative to mRNA species			
	6 (N)		1 (genomic)	
	Beaudette	M41	Beaudette	M41
1	0.13	0.13	1	1
2	0.15	0.13	1	1.2
3	0.23	0.23	1.8	1.8
4	0.41	0.28	2.2	3.2
5	0.62	0.17	1.3	4.8
6	1.00	1.00	7.7	7.7

RNAase A treatment of IBV virions

Allantoic fluid from 65 eggs infected with IBV-M41 was harvested, clarified, and the virus pelleted at 35,000 g for 1.5 hr. The virus was resuspended in 1.2 ml of NET buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) and divided into 90- μl aliquots. Some aliquots were then incubated at 37° for 5 or 30 min with RNAase A (Sigma, grade I-A). Virions in other samples were first treated with 2% Nonidet P-40 nonionic detergent (BDH, Poole, UK) to dissolve the virus envelope before addition of RNase. Controls were non-treated virus simply incubated at 37° for 5 and 30 min and virus incubated with NP-40 after addition of 1 μl (20 units) of RNAGuard RNase inhibitor (Pharmacia). The RNA was extracted by adding 2 ml of solution D (Chomczynski and Saachi, 1987), 5 μg of yeast tRNA was added (Sigma, type X-SA), and the RNA purified. Half of each sample, equivalent to the virus from about 2.5 eggs, was used for electrophoresis.

RESULTS

Molar ratios of intracellular IBV RNAs

Prior to this investigation the mRNAs of only one IBV strain, Beaudette, had been described (Stern and Kennedy, 1980). Also the molar ratios established for Beaudette were based on estimates of the M_r of the mRNAs deduced from agarose gels whereas much more accurate values were available to us following the complete sequencing of the IBV genome (Bourne et al., 1987). The molar ratios of the viral RNAs in terms of the smallest mRNA, number 6, is shown in Table 1 and were determined from two ^{32}P -labeled preparations of each of Beaudette and M41 (Fig. 1). Our results for Beaudette differed from those of Stern and Kennedy (1980) for mRNAs 1–3, our estimates indicating two- to threefold greater amounts of these RNAs, while being virtually identical for RNAs 4–6. The higher amount of RNA 3 determined by us was not simply due to the use

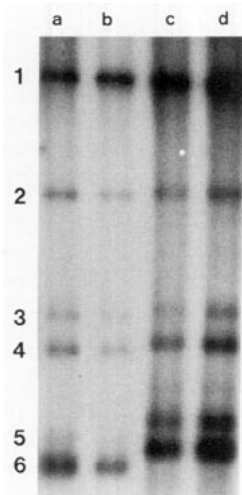


FIG. 1. Agarose gel electrophoresis of RNA extracted from CK cells infected with (a,b) IBV-M41 and (c,d) IBV-Beaudette in the presence of ^{32}P . The RNA for (a) and (c) was obtained using a batch of CK cells distinct from that used for (b) and (d) and at a different time. Band 1 is the gRNA, bands 2 through 6 are the viral subgenomic mRNAs.

of a more accurate size estimate of this RNA. Visual inspection of Fig. 1 and comparison with the results of Stern and Kennedy (1980) show that our strain of IBV-Beaudette produced more RNA 3 than the Beaudette strain of Stern and Kennedy. IBV-M41 differed from Beaudette in clearly having about fourfold less mRNA 5. The mRNAs of M41 were all smaller than those of Beaudette which reflects that Beaudette has an additional 184 bases in the nontranslated region downstream from the N gene (Boursnell *et al.*, 1985).

The cell-associated viral RNAs of two other strains of IBV (NL/D1466/78 and UK/123/82), with substantial sequence differences from Beaudette and M41, were analyzed by probing Northern blots with an N gene-specific probe. The mRNA profiles were not quantified but were broadly similar to those of the other two strains, the sizes of the mRNAs being indistinguishable from those of Beaudette (data not shown). These and preparations of many other strains of IBV (not shown) frequently had one or two additional bands which hybridized with the N gene probe, as seen for Beaudette (Fig. 2) and M41 (Figs. 3 and 6). The band (6.5 kb for Beaudette) between mRNAs 2 and 3 hybridized 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 (2.7 kb for Beaudette), between mRNAs 4 and 5 did not bind any of the pol and S gene probes (data not shown).

Detection of IBV mRNAs in virions

RNA extracted from virions of Beaudette included mRNAs (Fig. 2, lane e). Comparison with that extracted from infected Vero and CK cells (Fig. 2, lanes b and d)

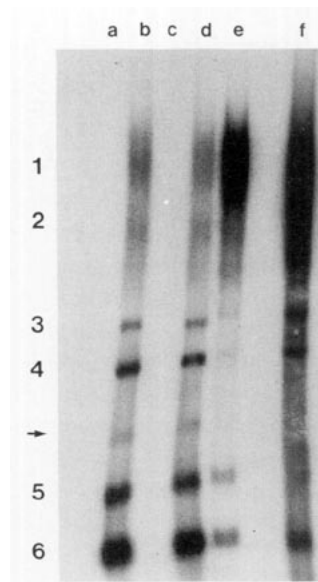


FIG. 2. Northern blot of IBV cell-associated RNAs, previously separated in an agarose gel, probed with a ^{32}P -labeled N' gene probe: RNA from mock-infected (a) Vero and (c) CK cells; cell-associated RNA from infected (b) Vero and (d) CK cells; virion-associated RNA from IBV strains (e) Beaudette and (f) UK/142/86. The arrow indicates an IBV RNA species which has not been identified as a functional mRNA.

clearly showed that the gRNA/mRNA ratio was much greater for virions than for intracellular RNA, indicating that the mRNAs had been incorporated at lower efficiency than gRNA. Virions of strain UK/142/86 also contained mRNAs (Fig. 2, lane f). The ratio gRNA/mRNA did not change with increased purification of

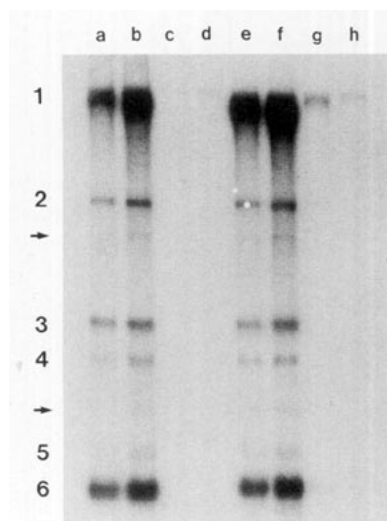


FIG. 3. Northern blot of IBV virion-associated RNAs, previously separated in an agarose gel, probed with a ^{32}P -labelled N gene probe, showing the continued presence of mRNAs during purification of M41 virions: (a) primary pelleted virions; (b) banded virions; (c-h) isopycnic sucrose density gradient fractions in the region of the virus peak. The arrows indicate two IBV RNA species which have not been identified as functional RNAs.

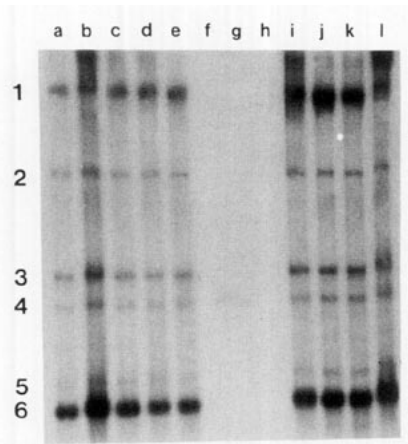


FIG. 4. Northern blot of IBV virion-associated RNAs, previously separated in an agarose gel, probed with a ^{32}P -labeled N gene probe, showing resistance of virion-associated mRNAs to RNAase A. RNA was extracted from M41 virions after incubation at 37° under the following conditions: (a and l) without either detergent Nonidet P-40 or RNAase; (b) with NP-40 but without RNAase; (c–e) five-minute incubation without NP-40 but with RNAase at (c) 0.1, (d) 1, and (e) $10\ \mu\text{g/ml}$; (f–h) as for (c–e), respectively, but with NP-40 to expose the RNAs previously within the virions; (i–k) as for (c–e), respectively, but longer (30-min) incubation.

virions (Fig. 3). Lanes c–h in Fig. 3 also show that after sucrose gradient sedimentation of M41 virions the fractions containing most mRNA 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 mRNAs with the virions.

To examine this point further virions were incubated with RNAase A, on the premise that the mRNA would be digested if it were external to the virions. Figure 4 shows that no viral RNA was destroyed at the highest concentration ($10\ \mu\text{g/ml}$, 30 min, 37°) of RNAase used whereas all RNA was destroyed when the virion membrane was dissociated with nonionic detergent.

Taken together these experiments showed that IBV virions contained viral mRNAs. To investigate whether cellular mRNAs had also been incorporated into virions, RNA from virions and from both infected and mock-infected CAM cells was probed with cDNA corresponding to the mRNA of chicken polypeptide chain elongation factor 1α (PCEF). This probe readily detected PCEF mRNA in CAM cells (Fig. 5A, lanes e and f; Fig. 5B, lane c) but no PCEF mRNA was detected in virions (Fig. 5A, lane d; Fig. 5B, lane b). When account had been taken of the proportions of cells infected, band intensities and autoradiograph exposure times, it was estimated that the amount of PCEF mRNA in infected CAM cells was approximately 25–50% of that of IBV mRNA 2 in CAM cells.

When Northern blots of virion RNA were probed with the negative- and positive-sense oligonucleotides (^{32}P -labeled) used to make the N gene cDNA by the PCR,

only the negative-sense probe detected IBV RNAs, showing that the great majority of the subgenomic RNAs were mRNAs (data not shown). Similarly, when a dilution series of extracts of Beaudette-infected CK and CAM cells were electrophoresed and probed, only the negative-sense probe hybridized to the viral RNAs (data not shown). Comparison of RNA dilutions and exposure times indicated that infected cells contained at least 100-fold more positive (mRNA)-sense subgenomic RNA than anti-mRNA-sense RNA.

Efficiency of incorporation of mRNAs into virions

The molar ratios of the RNAs in virions was estimated from the amount of N gene probe bound to Northern blots. The 3' coterminal 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 radiolabeled IBV RNA) compared with almost 100% for the mRNAs. The molar ratio of M41 RNAs was estimated from blots of dilutions of virion RNA probed with an ECL probe (Fig. 6). Comparison of band intensities indicated that the molar ratio gRNA/mRNA ranged from about 25 for mRNA 6 to 800 for mRNA 5. When account was taken of the molar ratios of gRNA/mRNA within cells (Table 1), i.e., in which most mRNAs outnumber gRNA, the efficiency of incorporation of gRNA was estimated to be greater than

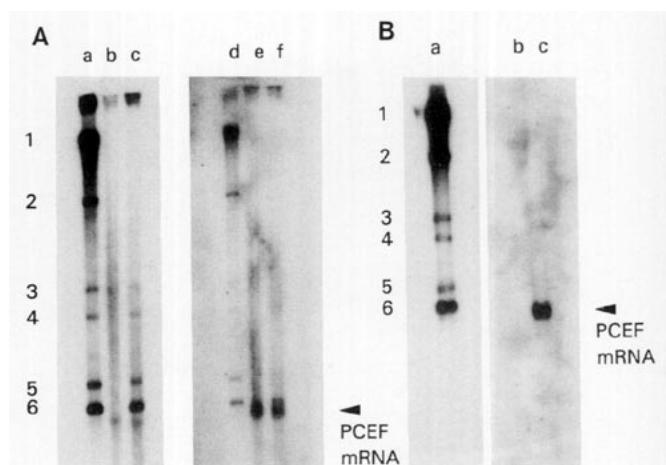


FIG. 5. Absence of the mRNA for chicken polypeptide chain elongation factor 1α (PCEF) in virions of IBV-Beaudette. (A) Virion-associated RNA (a), mock-infected (b), and IBV-infected (c) CAM RNA was electrophoresed in an agarose gel and transferred to nitrocellulose. After probing with a ^{32}P -labeled N gene probe (a–c) most but not all of the probe was removed by soaking the blot in hot 0.1% SDS and the blot reprobed with the PCEF probe (d–f). (B) Virion-associated RNA probed with (a) IBV N gene probe and (b) chicken PCEF probe. Lane (c) shows CAM RNA probed with the PCEF probe.

mRNA 2 by about 100-fold ranging to about 500-fold in respect of mRNA 5 (Table 2). The molar ratio for RNAs in virions of Beaudette was calculated from blots probed with a ^{32}P -labeled probe, the gRNA/mRNA ratio ranging from approximately 10 for mRNA 6 through to 30 for mRNAs 3 and 4. The efficiency of incorporation of gRNA was approximately 20-fold greater than for mRNA 2 and 100-fold greater than mRNAs 3 and 4.

DISCUSSION

Our results show that the virus-specific, subgenomic RNAs associated with preparations of gRNA derived from IBV virions are predominantly of mRNAs and were within virions. The amounts of mRNAs within the IBV virions, relative to gRNA (Table 2), are more than have been reported for MHV but less than for TGEV and BCV. Sethna *et al.* (1991) have reported that virions of TGEV contained 5- to 14-fold more gRNA than specified mRNAs, the N protein-encoding mRNA being the most abundant. Bovine coronavirus virions have been reported to contain more molecules of N and M protein-encoding mRNAs than gRNA (Hofmann *et al.*, 1990). The majority of IBV virions probably do not contain any mRNA species. In the case of IBV-Beaudette the ratio gRNA/mRNA in virions ranged from 8 to 32, depending on the species of mRNA. Assuming an average gRNA/mRNA ratio of 16 then one might expect 1 in 16 virions to contain a specified mRNA. Since there are five mRNAs then approximately 1 in 3 virions might have any one of the mRNAs. Virions of IBV-M41 contained less mRNAs (Table 2) than Beaudette, lead-

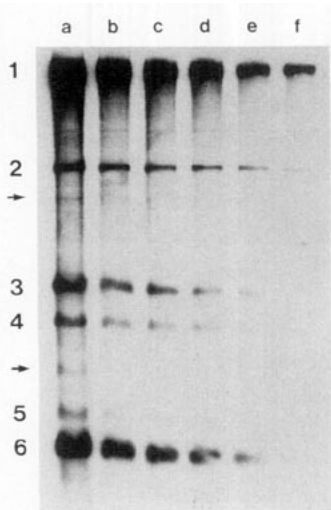


Fig. 6. Northern blot of IBV virion-associated RNAs, previously separated in an agarose gel, probed with a N gene probe detected using the enhanced chemiluminescence method. The RNA was extracted from an amount of virions equivalent to that produced in (a) 2, (b) 1, (c) 0.5, (d) 0.25, (e) 0.12, and (f) 0.05 of an embryonated fowl egg. The arrows indicate two IBV RNA species which have not been identified as functional mRNAs.

TABLE 2

EFFICIENCY OF INCORPORATION OF IBV GENOMIC AND SUBGENOMIC RNAs INTO VIRIONS

mRNA species	Reciprocal of molarity relative to RNA 1 in virions	Molarity relative to RNA 1 in cells	Reciprocal of relative efficiency of incorporation into virions
1	1	1	1
2	96	1	96
3	96	1.8	173
4	192	2.2	422
5	384	1.3	499
6	24	7.7	185

ing to an estimate of 1 in 20 virions having any one of the mRNAs.

The efficiency of incorporation of mRNAs into IBV virions was some two orders of magnitude less than that of gRNA (Table 1). The frequency of occurrence of mRNAs within virions is higher than one would expect from this great difference because there is a greater number of subgenomic mRNA molecules than gRNA in infected cells (Table 2). The much greater efficiency of packaging of gRNA than mRNA is consistent with observations that a sequence near the 3'-end of MHV gene 1, within open reading frame 1b, is essential for high efficiency incorporation of RNA into virions of MHV (Makino *et al.*, 1990; van der Most *et al.*, 1991; Fosmire *et al.*, 1992). None of the subgenomic mRNAs of IBV contain any part of gene 1, although they do possess a 63-base leader sequence derived from the 5'-terminus of the gRNA (Brown *et al.*, 1984; Bournsnel *et al.*, 1987). The amount of chicken PCEF mRNA in infected CAM cells was less than that of IBV mRNA 2 and more than the 6.5-kb IBV RNA species. However, whereas both of these IBV RNAs were present in virions, no PCEF mRNA was detected. This indicates preferential incorporation of viral RNAs into virions. Further studies will be required to determine whether the 5'-terminal leader sequence and/or 3'-terminal sequences, which are common to gRNA and all the subgenomic mRNAs, play any role in the low efficiency incorporation of subgenomic viral mRNAs into virions.

What role, if any, might intravirion mRNAs have in the replication cycle of coronaviruses? Available evidence indicates that the major mechanism by which mRNAs are generated is that of a process of discontinuous transcription (Baric *et al.*, 1983; Yokomori *et al.*, 1992). It is envisaged that transcription of anti-genome first produces a leader sequence which dissociates from the template and reassociates downstream at homologous sequences at the beginning of each gene after which transcription of the body of the mRNAs occurs. As cells infected with TGEV and BCV contain negative-sense copies of the mRNAs, it has been suggested

that coronavirus mRNAs might function as replicons (Sethna *et al.*, 1989; Sawicki and Sawicki, 1990; Hofmann *et al.*, 1990; Sethna *et al.*, 1991). It is conceivable, therefore, that mRNAs within virions might, upon their release from virions following penetration of host cells, be replicated. The results of a study of ultraviolet light irradiation during MHV replication supported a discontinuous transcription mechanism as being the major means by which mRNAs are generated but allowed for the possibility that once mRNAs had been synthesized in this way that subsequent synthesis of some mRNAs, perhaps a minor amount, could occur as a result of mRNAs acting as replicons (Yokomori *et al.*, 1992). Some such replication of mRNAs might be expected since coronavirus mRNAs have 5' and 3' sequences which are identical to those of gRNA (reviewed by Spaan *et al.*, 1988; and Lai, 1990). Since most IBV virions do not contain any mRNA, let alone a copy of each mRNA, it is highly unlikely that the incorporation of mRNAs into virions is a necessary facet of coronavirus replication. Nevertheless there might be some consequences of the presence of mRNAs in virions.

Some coronavirus particles would, in effect, have two copies of one gene 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.* (1991) have shown that when MHV-infected cells were transfected with a subgenomic DI RNA, which possessed a leader sequence, an intergenic region associated with leader recognition and an open reading frame (ORF), synthesis of the subgenomic DI did not occur. In contrast, transfection of infected cells with a genomic DI which also contained elements of gene 1 did result in the production of the subgenomic DI. This, plus the ultraviolet irradiation study (Yokomori *et al.*, 1992), suggests that the role played by virion-associated mRNA would be small, i.e., that most of the mRNA synthesised *de novo* would arise from transcription of full-length anti-genome rather than from negative-sense subgenomic RNA derived from the incoming mRNA.

Another way in which virion-associated mRNA might have a biological effect is in respect of recombination. This has been demonstrated experimentally for MHV and is believed to occur by a process of template-switching (Lai *et al.*, 1985; Makino *et al.*, 1986). Recombination would appear to be able to occur during synthesis of both positive- and negative-sense RNA and recombination between replicating MHV gRNA and nonreplicating subgenomic RNA has been demonstrated (Koetzner *et al.*, 1992; Liao and Lai, 1992). Sequence analysis of field strains of IBV has provided circumstantial evidence that IBV undergoes recombina-

tion (see Introduction for references). Recombination is most likely to occur during mixed infection. In addition, some virions might contain heterologous mRNA. Infection of another cell, in the same or another chicken, might result in recombination between the gRNA and heterologous subgenomic RNA. The potential for mixed infection and hence recombination of IBV in the field is high. The virus is ubiquitous and highly contagious. Billions of doses of live virus vaccine doses are used, despite which field isolates abound, including coexistence of distinct isolates, and virus can persist in individual birds and be reexcreted at around point-of-lay (Jones and Ambali, 1987).

In conclusion, IBV mRNAs were incorporated into virions of IBV, at low efficiency, and might play a minor role during virus replication.

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