A Leader Sequence is Present on mRNA A of Avian Infectious Bronchitis Virus

By T. D. K. BROWN,* M. E. G. BOURSNELL AND M. M. BINNS
Houghton Poultry Research Station, Houghton, Huntingdon, Cambs. PE17 2DA, U.K.

(Accepted 27 April 1984)

SUMMARY

The subgenomic mRNAs of the fowl coronavirus infectious bronchitis virus (IBV) form a 3' co-terminal or 'nested' set. The RNase T1 oligonucleotide mapping data which demonstrated this form of sequence organization provided no evidence for the presence of non-contiguous (leader) sequences fused to the 5' termini of the mRNAs. However, we have been able to demonstrate, by extension of an end-labelled synthetic oligonucleotide primer, the presence of a leader sequence on IBV mRNA A. Sequencing by the chemical degradation method of the extended product has yielded the almost complete sequence of the 60 base leader and its point of fusion to the sequence of the virus genomic RNA.

Infectious bronchitis virus (IBV) is a coronavirus which infects the domestic fowl and is capable of causing economically important disease in intensively reared birds. IBV virions are enveloped and pleomorphic; they contain three major virus-coded protein structures, the surface projection glycoprotein, the membrane protein and the nucleocapsid protein (Cavanagh, 1981; Stern et al., 1982). The viral genome is a positive-stranded RNA molecule of approximately 20 kilobases (Stern & Kennedy, 1980b). Six polyadenylated viral mRNA species (A to F) have been detected in infected cells. mRNA A is the smallest and mRNA F is of genome length (Stern & Kennedy, 1980b). These mRNAs form a so-called 'nested' or 3' co-terminal set (Fig. 1a) (Stern & Kennedy, 1980a, b); evidence from translation studies in vitro suggests that the mRNAs are translated to give a single major polypeptide. Thus, mRNA A codes for the nucleocapsid polypeptide, mRNA C for the membrane polypeptide and mRNA E for the precursor of the surface projection polypeptides (Stern et al., 1982; Stern & Sefton, 1982b, 1984). The sizes of the translation products are consistent with the coding capacity present at the 5' end of each mRNA, but not present in the next smallest mRNA. U.v. inactivation studies have demonstrated that the subgenomic mRNAs are not produced by processing of larger RNA species (Stern & Sefton, 1982a).

There is therefore a basic understanding of the replication strategy of IBV. However, work with the mouse coronavirus, murine hepatitis virus (MHV), has demonstrated certain unusual features of subgenomic RNA synthesis in this system. RNase T1 oligonucleotide mapping studies of MHV mRNAs and genomic RNA revealed minor discrepancies in the 3' co-terminal set model (Lai et al., 1981, 1982; Leibowitz et al., 1981). These differences have been explained in terms of the presence of leader sequences derived from the 5' terminus of the viral genome at the 5' terminus of each subgenomic mRNA (Lai et al., 1983). These leaders are fused to the body sequences of the mRNAs which are derived from the position in the genome expected from the 3' co-terminal set model. This view has been confirmed and extended by sequencing studies of cDNA clones of mRNA and direct sequencing of genomic RNA and mRNA (Spann et al., 1983). Conserved sequences have been detected in the genomic sequence at the proposed fusion site for mRNA 7 (Spann et al., 1983).

The RNase T1 oligonucleotide mapping experiments which had so convincingly established the 3' co-terminal set model for the sequence organization of IBV subgenomic RNAs did not demonstrate the existence of a similar leader fusion mechanism in IBV subgenomic RNA.
synthesis. This suggested the possibility that there could be a major difference in the replication strategies of IBV and MHV. We have therefore investigated the existence of fused non-contiguous sequences on IBV mRNA A and their relationship to the previously detected regions of homology present in IBV genomic RNA close to mRNA boundaries (Brown & Boursnell, 1984; Boursnell et al., 1984). This would have the merit of establishing whether the leader fusion mechanism is a general feature of coronavirus subgenomic RNA synthesis and also illuminating more clearly features of sequence organization potentially involved in such a mechanism.

IBV strain Beaudette was used throughout. Primary chick kidney (CK) cell cultures were prepared essentially as described by Youngner (1954).

The isolation and characterization of genomic cDNA clones has been described in detail previously (Brown & Boursnell, 1984). Clone C5.136 which covers the region between 1.0 and 3.3 kb from the 3' end of the genomic RNA was used in this study.

Cytoplasmic RNA from IBV-infected CK cultures was isolated as follows. Twenty 90 mm Petri dishes of confluent CK culture were infected with approximately 2 x 10⁷ p.f.u./dish of IBV in 5 ml serum-free Eagle's MEM. After 1.5 h at 37 °C the virus inoculum was removed and replaced with 10 ml MEM containing 1% inactivated calf-serum. After a further 16 h at 37 °C the supernatant was removed, the cell sheets washed with ice-cold phosphate-buffered saline and the total cytoplasmic RNA extracted using an NP40 lysis technique. This was carried out as described by Maniatis et al. (1982) except that 10 mM-vanadyl ribonucleoside complex (VRC) (Berger & Birkenmeier, 1979) was included in the lysis buffer. The proteinase K-treated material was extracted twice with phenol/chloroform/methylbutanol (50:49:1, by vol.) containing 0.01% 8-hydroxyquinoline, ethanol-precipitated with 2.5 vol. ethanol, dried, dissolved in 10 mM-VRC
and re-precipitated with 2.5 vol. ethanol. Prior to use, the RNA was pelleted, dried, dissolved in sterile water, extracted twice with phenol/chloroform, re-precipitated with ethanol, dried and finally dissolved in 20 μl sterile water.

Primer extension on IBV mRNA A with a synthetic oligonucleotide primer was carried out as follows. A 15 base synthetic oligonucleotide primer (5' GGCTCTGCTTGTCTCCT 3') (Cruachem) complementary to the genomic RNA 3'-wards of the region of homology at the 5' terminus of mRNA A (Fig. 1b) (Brown & Boursnell, 1984) was 5' labelled with [γ-32P]ATP using polynucleotide kinase. Fifty ng of primer were incubated with 100 μCi [γ-32P]ATP (3000 Ci/mmol) in 50 mm-Tris-HCl pH 7.6, 10 mm-MgCl2, 1 mm-spermidine in a volume of 40 μl for 15 min at 37 °C with 20 units polynucleotide kinase. The reaction mixture was passed over a Sephadex G-25 column equilibrated with sterile water and the excluded fractions pooled and freeze-dried. The labelled primer was used to prime reverse transcription in a total cytoplasmic RNA preparation from IBV-infected CK cultures (see above). The reaction was carried out in 150 mm-KCl, 50 mm-Tris-HCl pH 8.3 (at 42 °C), 10 mm-MgCl2, 20 mm-dithiothreitol containing 35 units of human placental RNase inhibitor (Biotec), 4 μl of RNA (see above), 1 mm-dNTPs and 28 units of reverse transcriptase (Life Sciences, St Petersburg, Fla., U.S.A.) for 30 min at 42 °C. An equal volume of deionized formamide containing 0.03% xylene cyanol FF, 0.03% bromophenol blue and 20 mm-disodium EDTA was added and the sample heated to 100 °C for 3 min. It was immediately loaded onto a 6% polyacrylamide sequencing gel and electrophoresed until the xylene cyanol was approximately two-thirds of the way down the gel. The gel was exposed to X-ray film and the labelled band corresponding to the extended primer identified and cut from the gel.

The labelled fragment was eluted from the polyacrylamide gel using essentially the technique of Maxam & Gilbert (1980), except that the elution buffer contained no magnesium acetate, and sequenced as described previously (Brown & Boursnell, 1984). The sequencing of the genomic cDNA clone was carried out in a similar manner.

The potential relationships of the synthetic oligonucleotide primer sequence to the sequences of IBV genomic RNA and IBV mRNA A are presented in Fig. 1(b). It can be seen that primer extension on a total population of IBV mRNAs would be expected to give rise to a relatively short extended product derived from priming on mRNA A, the most abundant mRNA species, and smaller amounts of extension products derived from the larger mRNAs. The expected product of extension on mRNA A was indeed observed (Fig. 2). The size of the fragment estimated from the observed position of the xylene cyanol marker was approximately 70 bases. The sequence of the extended primer fragment would be expected to include an element of genomic sequence including the region of homology previously demonstrated to lie at the 5' end of mRNA A or at the 5' end of the body of mRNA A. If no leader sequence was present on the mRNA this genomic sequence would continue to the 5' end of the RNA. If, however, a leader sequence was present a divergence from the genomic sequence would be expected at, or close to, the region of homology. An autoradiograph of a gel used to sequence the primer extension fragment is presented in Fig. 2. The sequence data derived from this and other gels and the corresponding region of genomic sequence are presented in Fig. 3. The expected origin of the priming event is confirmed by the identity with genomic sequences, including part of the region of homology, at the 3' end of the sequence, with divergence occurring in the region of homology and extending 5'-wards. These data demonstrate the presence of a leader sequence of approximately 60 bases on IBV mRNA A. It proved impossible to identify unequivocally four of the last six bases at the 5' terminus of the leader.

This observation of a leader sequence supports the generality of the mechanism of subgenomic RNA synthesis among the Coronaviridae. The sequence to which the leader is joined (5' CTTAACAA 3') is the most highly conserved element of the three regions of homology so far identified in IBV genomic RNA; these regions lie at the boundaries of mRNAs A, B and C (Brown & Boursnell, 1984; Boursnell et al., 1984). The positioning of the leader/body junction within the region of homology is also a feature of MHV mRNA synthesis (Spaan et al., 1983). The 60 base leader sequence of IBV mRNA A has a low content of G (12%) and C (12%) with a high A (32%) and U (37%) content, with 7% of the sequence at the 5' terminus undetermined.
There is considerable potential for the formation of secondary structure within the leader and also between the leader and the 5' part of the body of mRNA A. This may be of significance in mRNA synthesis.

The sequence of the leader and the 5' terminus of the body of mRNA A predicts two relatively large RNase T1 oligonucleotides which might have been expected to be detected in the studies of Stern & Kennedy (1980a, b). The larger of these, a 26 base oligonucleotide, is contained wholly within the leader sequence. If leader sequences are present on all subgenomic mRNAs this
would be seen in digests of mRNAs and also genomic RNA; it could thus appear to be simply a 3' terminal oligonucleotide. However, it should only have been observed in the largest of the size-fractionated pools of alkali-fragemented genomic RNA which had been selected on oligo(dT)-cellulose (Stern & Kennedy, 1980b). It is likely that the failure to observe this particular difference in the oligonucleotide maps was the result of technical difficulties in resolving the complex mixture of spots. The smaller of the predicted oligonucleotides is 18 bases long and spans the leader/body junction. It is possible that this oligonucleotide was detected by Stern & Kennedy (1980a) when they reported the presence of anomalous spots in the RNase T1 fingerprints of mRNAs A and C.

Various models can be advanced to explain the fusion of leader sequences to mRNA bodies during coronavirus mRNA synthesis in the absence of 'classical' splicing which is ruled out by u.v. target size data (Stern & Sefton, 1982a). Two broad classes of model can be distinguished: (i) those involving independent synthesis of leader and body followed by fusion, and (ii) those in which the leader RNA is required to prime synthesis of the body and is fused to it during synthesis. Within the primer model it is possible to envisage two types of mechanism. In one case the leader remains part of the replicative intermediate (RI) with the negative strand template looping out and synthesis continuing at the selected initiation site on the template and in the other the primer is selected from a 'free' pool of leader RNA by a polymerase molecule bound solely at the initiation site for synthesis of the mRNA body.

Certain features of potential mechanisms for MHV mRNA synthesis based on sequence data and analogies with other viral systems have been discussed (Spaan et al., 1983). However, recent work on the RI RNA of MHV has shed more direct light on the mechanism of mRNA synthesis (Baric et al., 1983). These data suggest that models involving leader priming with looping out of the template or post-transcriptional fusion are less likely than those involving 'free' leader-primed transcription.

The mechanism by which the initiation sites for synthesis of mRNA bodies are recognized by the viral polymerase requires further characterization. It is possible that this involves base pairing between the leader RNA and the negative strand template. It will be difficult to test this idea directly without the isolation and sequencing of molecules from the putative 'free' leader pool. If this mechanism operates in a straightforward manner in IBV mRNA synthesis it would suggest, however, that two distinct forms of mRNA B could be made as the highly conserved sequence of homology is duplicated in the genome at the 5' terminus of the body of mRNA B. It is not, of course, necessary to invoke base pairing as the RNA polymerase may simply have affinity for the sequences of the regions of homology.

It is likely that analysis of the mechanism of IBV mRNA synthesis will involve sequencing of the 5' terminus of genomic RNAs and further junction regions and also studies of the viral RNA polymerases, leader and RI RNAs.
Short communication

We are grateful to Anne Foulds, Ian Foulds, Penny Gatter and Bridgette Britton for excellent technical assistance. This research was carried out under Research Contract No. GBI-2-011-UK of the Biomolecular Engineering Programme of the Commission of the European Communities.

REFERENCES


(Received 20 February 1984)