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Avian infectious bronchitis virus genomic RNA contains sequence homologies at the intergenic boundaries

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(Accepted 21 November 1983)

Summary

cDNAs derived from the 3'-terminus of IBV Beaudette genomic RNA by oligo(dT)-primed reverse transcription were cloned in PAT153/HB101. 25 clones were characterised and two of these were found to cover 3.3 kb extending from the poly(A) tail. The relationships of the two smallest IBV mRNAs, A and B, to the cloned sequences were established by restriction fragment hybridisation to Northern blots. The 5'-termini of mRNAs A and B were located on the clones by S1 mapping. DNA sequencing of cloned sequences corresponding to the intergenic boundaries of mRNAs A and B was carried out. Homologies between the sequences at these boundaries were observed.

infectious virus bronchitis Beaudette, gene cloning, intergenic sequences

Introduction

The genome of a coronavirus is typically an RNA of 15–20 kilobases (kb) with positive polarity (Siddell et al., 1983). A series of viral mRNAs have been identified in infected cells (Siddell et al., 1983). Thus six major species of viral RNA have been observed in IBV-infected cells; they have been designated A–F with A being the smallest and F, which is the same length as virion RNA, being the largest (Stern and Kennedy, 1980a). The gross sequence relationships between the genomic RNA and subgenomic mRNAs have been established for IBV using the technique of oligonucleotide mapping (Stern and Kennedy, 1980b). These data demonstrate that the
mRNAs are a 3'-coterminal ('nested') subset of the genomic RNA sequence. The assignment of polypeptide coding to the mRNAs by in vitro translation studies has lent support to the idea that each mRNA gives rise to one major primary translation product encoded by the 5' sequences of each mRNA which are not present in the smaller mRNA species and that the other coding regions 3'-wards of this region are not translated at high efficiency (Stern et al., 1982). Studies of UV inactivation of viral mRNA synthesis have thrown light on the basic mechanism by which the subgenomic RNAs are produced during IBV replication; they are synthesized independently rather than by cleavage of a genome-length precursor (Stern and Sefton, 1982). Recently, oligonucleotide mapping of mRNAs, sequencing of cDNA clones of mRNAs and direct sequencing of mRNAs and the genome of another coronavirus, mouse hepatitis virus (MHV) have demonstrated that mRNA synthesis involves fusion of a 5'-terminal 'leader' sequence from the genomic RNA to the 'body' of each subgenomic mRNA (Lai et al., 1982, 1983; Spaan et al., 1982, 1983). The oligonucleotide mapping data obtained for IBV mRNAs (Stern and Kennedy, 1980b) do not provide evidence for the existence of 'leader' sequences.

These observations have prompted our investigations of the sequences present in the genomic RNA of IBV Beaudette in the areas corresponding to the 5'-termini of mRNAs A and B. We have cloned cDNA prepared by oligo(dT) priming of IBV genomic RNA in pAT153/HBl01 and characterised clones covering approximately 3.3 kb extending from the poly(A) tract at the 3'-terminus. The 5'-termini of the mRNAs have been mapped onto the genomic clones and the relevant regions in the clones sequenced.

Materials and Methods

Isolation of cDNA clones

IBV strain Beaudette was grown in 11-day-old embryonated eggs. Virions were isolated from allantoic fluid and purified by isopycnic centrifugation on sucrose gradients. RNA was isolated from purified virions by incubation with proteinase K (500 µg/ml) in 0.1 M Tris-HCl (pH 8.0)/0.5% SDS for 1 h at 37°C followed by repeated extractions with phenol/chloroform/isoamyl alcohol (50 : 49 : 1, v/v) containing 0.1% hydroxyquinoline. The RNA was ethanol precipitated, dissolved in water and reprecipitated with ethanol for storage. 60 µg of viral RNA was reverse transcribed into DNA for 1 h at 42°C in a volume of 1 ml containing 50 mM Tris-HCl pH 8.3, 50 mM KCl, 10 mM MgCl₂, 4 mM dithiothreitol, 20 µg oligo(dT), 0.5 mM dATP, dGTP, dTTP, 0.2 mM dCTP, 10 µCi α-[³²P]dCTP, 4 mM sodium pyrophosphate and 500 units of AMV reverse transcriptase (a gift from Dr. J.W. Beard). The reaction was terminated by addition of SDS to 0.1% and EDTA to 5 mM and extracted with phenol/chloroform. RNA was destroyed by incubation with 0.1 M NaOH for 20 min at 70°C. The mixture was neutralised with acetic acid and passed over a Sephadex G-100 column. Excluded fractions were pooled, ethanol precipitated and reverse transcribed with 100 units of reverse transcriptase for 2 h at 44°C in a volume of 200 µl. After phenol/chloroform extraction the reaction
mixture was passed over Sephadex G-100 and the excluded fractions pooled and ethanol precipitated. They were then treated with 5 units/ml of nuclease S1 in 0.3 M NaCl, 30 mM sodium acetate, 3 mM zinc acetate pH 4.5 for 30 min at 37°C. The reaction mixture was adjusted to 5 mM EDTA, 0.1 M Tris-HCl pH 8.0 and phenol/chloroform extracted prior to passage over Sephadex G-100. The excluded fractions were once again ethanol precipitated.

The ds cDNA was tailed with dC residues using terminal transferase according to the procedure of Roychoudhury and Wu (1980). The tailed cDNA was annealed with a dG-tailed PstI digest of the plasmid pAT153 (Twigg and Sherratt, 1980) and the mixture used to transform *E. coli* HB101 to tetracycline resistance. Ampicillin-sensitive colonies were selected for further characterisation.

**Characterisation of cDNA clones**

A series of clones with relatively large inserts were identified by electrophoresis of colony lysates on agarose gels (Eckhardt, 1980). These clones were characterised by agarose gel electrophoresis of PstI digests, Southern blotting (Southern, 1975) onto nitrocellulose filters and hybridisation with a series of probes. The viral origin of the inserts was demonstrated by hybridisation with a probe prepared by polynucleotide kinase labelling of alkali-treated, full-length IBV genomic RNA. The absence of plasmid sequences interspersed with viral sequences was confirmed by hybridisation with nick-translated pAT153. The presence of poly(A) sequences from the 3′-terminus of the virus genome in cloned sequences was detected using polynucleotide kinase-labelled alkali-treated poly(U). The end of the clone bearing the cloned poly(A) sequences was identified by blotting and poly(U) hybridisation of restriction digests of appropriate clones. Ultimately restriction sites were mapped in 25 of the clones and this enabled the construction of a continuous map, 3.3 kb in length, which included the 3′-terminus of the viral genome.

**RNA blot hybridisation**

Total cytoplasmic RNA was purified from IBV-infected chick kidney monolayers using an NP40 lysis procedure, fractionated on 1.5% formaldehyde–agarose gels and transferred to nitrocellulose filters (Maniatis et al., 1982). Filters were probed with nick-translated IBV clone DNA and with nick-translated restriction fragments purified from clone inserts. The restriction fragments were purified by electrophoresis on either low gelling temperature agarose or polyacrylamide gels (Maniatis et al., 1982). Fragments were eluted from the agarose by melting and phenol extraction (Maniatis et al., 1982). They were eluted from the acrylamide gels as described by Maxam and Gilbert (1980) and further purified by binding to and elution from ‘Elutips’ (Schleicher and Schuell) prior to nick translation. Hybridisations were carried out for 16 h at 42°C in 50% formamide, 5 × SSPE, 1 × Denhardt’s solution, 100 µg/ml denatured, sonicated herring sperm DNA, 10% (w/v) sodium dextran sulfate 500 and 0.3% SDS using approximately 10^7 dpm/filter (10^8 dpm/µg). Filters were washed four times at room temperature in 10 mM sodium phosphate (pH 6.8)/0.2% SDS/1 mM EDTA and exposed at −70°C to preflashed Kodak XAR film using Dupont Lightning Plus intensifying screens.
S1 mapping

The PstI/PvuII (1462 bp) fragment of the C5.136 insert (see Fig. 1a) was purified on 3.5% polyacrylamide gels as described above. Approximately 500 ng of the fragment were 5'-labelled with γ-[\(^{32}\)P]ATP using polynucleotide kinase in 20 μl of 60 mM Tris-HCl (pH 7.8)/15 mM DTT/10 mM MgCl\(_2\) for 30 min at 37°C. S1 mapping was carried out using this fragment and total cytoplasmic RNA from IBV-infected chick kidney cultures. The modification of the Berk and Sharp procedure (1972) described by Weaver and Weissmann (1979) was used. Typically approximately 200 ng of labelled fragment was annealed with 0 or 500 μg of total cytoplasmic RNA in a volume of 30 μl under conditions favouring the formation of RNA/DNA hybrids (Casey and Davidson, 1977). 10 mM vanadyl ribonucleoside complex (Berger and Birkenmeier, 1979) was included to minimise RNA degradation. The hybridisation reactions were worked up as described (Maniatis et al., 1982) and treated with 500 U/ml of S1 nuclease at 37°C for 1 h. Protected \(^{32}\)P-labelled fragments were analysed on 5% non-denaturing polyacrylamide gels. Autoradiography was carried out as described above. The fragment sizes were estimated from their mobilities relative to those of \(^{32}\)P-end-labelled φX174 RF DNA/HaeIII fragments.

DNA sequence determination

Plasmid DNA was prepared by a modification of the method of Holmes and Quigley (1981). DNA restriction fragments, 3'-end-labelled with α-[\(^{32}\)P]dNTPs (> 3000 Ci/mmol) using Klenow polymerase, were sequenced essentially as described by Maxam and Gilbert (1980). The depurination reaction was carried out in 2% diphenylamine/66% formic acid/1 mM EDTA for 10 min at 20°C, followed by 3-fold dilution in water, three ether extractions and lyophilisation. Piperidine hydrolysis was carried out as previously described (Maxam and Gilbert, 1980). For the sequencing of some regions of the DNA, restriction digests of the viral insert were recloned into the plasmid pUC9 allowing sequencing from adjacent vector restriction sites (Messing and Vieria, 1982). Sequence data were stored and analysed on an Apple IIe microcomputer using the programs of Larson and Messing (1982).

Results

Location of intergenic boundaries in the genomic RNA of IBV Beaudette

In order to determine the location of the 5'-termini of the mRNAs in IBV genomic RNA it was first necessary to prepare a restriction map of appropriate clones covering the 3.3 kb cloned from the 3'-terminus of the viral genomic RNA. Two clones spanning the 3.3 kb were chosen for detailed study. The restriction sites and relationships of the clones are shown in Fig. 1a. Two approaches were used to locate the intergenic boundaries.

(i) Hybridisation of labelled restriction fragments to mRNAs. It was first necessary to determine the gross relationship between the cloned sequences and IBV mRNAs.
The mRNAs form a 3'-coterminal set which is also coterminous with the genomic RNA (Stern and Kennedy, 1980b). Molecular weight estimates of the viral mRNAs suggest that the 5'-termini of mRNAs A and B would lie within the cloned sequences, while the 5'-terminus of mRNA C would lie beyond the cloned region (Stern and Kennedy, 1980a, Fig. 2). This view is supported by the observation in primer extension experiments using mRNAs of a major band which maps approximately 350 bases beyond the 5'-end of the cloned sequences (Brown and Boursnell, unpublished). These predicted relationships between the 5'-termini of the mRNAs and the genomic RNA (and clones derived from it) are presented in Fig. 1b.

In order to demonstrate that the sequences present in the clones were organised in a way consistent with the 3'-coterminal set and to locate the approximate positions of the 5'-termini of mRNAs A and B, labelled restriction fragments purified from the insert of clone C5.136 were used to probe total cytoplasmic RNA from IBV-infected chick kidney cells, which was fractionated on formaldehyde agarose gels and transferred to nitrocellulose filters. These data are summarised for mRNAs A, B, C and D in Table 1. Data for the PvuII/PstI and AccI/AccI, AccI/PstI fragments are presented in Fig. 2. It can be seen that the fragments derived from the end of the C5.136 insert which is nearest to the 3'-end of the viral genomic RNA invariably hybridise to all mRNA species, while the fragments derived from the 5'-end of the insert hybridise only to mRNAs C and D. Thus both the PvuII/PstI fragments hybridise to all four mRNAs while the AccI/AccI fragment hybridises to mRNAs B, C and D, and the 940-bp AccI/PstI fragment hybridises to mRNAs C...
TABLE 1
HYBRIDISATION OF RESTRICTION FRAGMENTS DERIVED FROM CLONE C5.136 TO IBV mRNAs A, B, C AND D

All fragments are from the restriction site to PstI sites at the insert boundary except AccI-390 which is AccI to AccI.

| mRNAs | A | B | C | D |
|-------|---|---|---|---|
| Pvu II | 3' 820 | + | + | + | + |
|        | 5' 1480 | + | + | + | + |
| Taq I  | 3' 980 | + | + | + | + |
|        | 5' 1320 | - | + | + | + |
| Acc I  | 3' 390 | - | + | + | + |
|        | 5' 940 | - | - | + | + |
| Bam H1 | 3' 1910 | + | + | + | + |
|        | 5' 390 | - | - | + | + |
| Hae III| 3' 2020 | + | + | + | + |
|        | 5' 280 | - | - | + | + |

Fig. 2. Hybridisation of nick-translated restriction fragments of C5.136 to Northern blots of IBV mRNAs.
1, PvuII/PstI 820 bp fragment; 2, PvuII/PstI 1480 bp fragment; 3, AccI/AccI 390 bp fragment; 4, AccI/PstI 940 bp fragment. Replicate strips were cut from a single nitrocellulose filter and hybridised with the labelled restriction fragments. Molecular weight estimates for the mRNAs were obtained from the positions of unlabelled ribosomal RNAs from chicken and E. coli.
and D. The data presented in Table 1 show that the 5'-terminus of mRNA A lies between the PvuII and TaqI sites while the 5'-terminus of mRNA B lies between the two AccI sites. This experiment thus establishes the gross relationship between the cloned sequences and IBV mRNAs A and B.

(ii) S1 mapping. In order to locate accurately the termini of the mRNAs it was necessary to use S1 mapping. The protection of an appropriate 32P 5'-end-labelled restriction fragment by hybridisation with total cytoplasmic RNA from IBV-infected cells was studied. The results are presented in Fig. 3. The lengths of the protected fragments estimate the positions of the 5'-termini of mRNAs A and B in relation to the restriction map of the cloned sequences. Thus the 5'-terminus of the 'body' of mRNA A lies approximately 128 bp 5'-wards from the PvuII site and the 5'-terminus of mRNA B lies approximately 431 bp 5'-wards from this site. In all S1 mapping experiments there was protection of the full length labelled restriction fragment (up to the 5'-end of the cloned sequences) without evidence of a protected species slightly shorter than this which might correspond to a 5'-terminus of mRNA C. It was thus possible to locate the 5'-termini of mRNAs A and B with sufficient accuracy to obtain sequence data and examine them for features of possible significance.

Sequencing of two intergenic boundaries in IBV genomic RNA
The regions demonstrated by S1 mapping to contain mRNA junctions were

![Fig. 3. S1 mapping of the 5'-termini of mRNAs A and B on cDNA clone C5.136 using a 5' 32P-labelled PstI/PvuII fragment purified from a polyacrylamide gel. The protected fragments obtained as described in Materials and Methods were analysed on a 5% Tris-borate–EDTA polyacrylamide gel. Molecular weights were estimated from the positions of the end-labelled HaeIII digest of φX174 RF.](image-url)
Fig. 4. Sequences of cDNA at the 5'-termini of mRNAs A and B. (a) The arrows show the positions of the 5'-termini of the mRNAs as determined by S1 mapping. The underlined regions show homologies between the two regions. (b) and (c) show two possible alignments of the two regions which give high degrees of homology.

 sequenced and the sequences obtained for them compared for homology using a 'matrix' approach (Maizel and Lenk, 1981). Extensive sequence homologies between the two predicted sites were indeed observed (Fig. 4). Thus a nine-base stretch of sequence is common to the two regions and is present twice at the end of mRNA B; other homologies are also observed.

Discussion

The results presented above establish the location of two intergenic boundaries on a cDNA clone derived from IBV genomic RNA. These are the first sequence data obtained from clones of coronavirus genomic RNA. The significance of the observation of a series of sequences repeated at these intergenic boundaries is unclear at the present time, but it seems likely that they are important in the synthesis of viral mRNAs rather than being involved in mRNA binding to ribosomes (Kozak, 1983). The finding that the synthesis of viral mRNAs in MHV infection involves fusion of a 5'-leader sequence to the mRNA bodies makes it possible that a similar mechanism operates for synthesis of viral RNAs in IBV infection. Direct sequencing of IBV mRNA suggests that this is also the case for IBV RNA synthesis (Brown and Boursnell, unpublished). It should be noted that if such leader sequences do exist in IBV, then the S1 mapping data provide the location of the 5'-termini of the 'bodies' of the mRNAs. These would then represent the points at which the mRNA sequences would diverge from the genomic sequences. The IBV sequences show no significant homology with the equivalent MHV sequence; however, low G content is common to both IBV and MHV boundary sequences. Only one boundary sequence
is available for MHV (derived from cDNA clones of mRNAs rather than genomic RNA) and it is thus impossible to make other types of comparison at the present time (Spaan et al., 1983). It is possible that the sequences may be involved in base pairing with sequences present in a putative leader sequence or that they are recognised directly by the viral RNA polymerase. The observation that a nine-base sequence present once at the A/B boundary is present twice at the B/C boundary suggests the possibility that the degree of iteration might control the rates of synthesis of viral RNAs by increasing the probability of polymerase binding at the repeated site. However, the observation that in cytoplasmic RNA preparations from IBV-infected cells the abundance of mRNA A is greater than that of B (Stern and Kennedy, 1980a) indicates that, if this is so, other factors must also be involved. It will be important to establish the relationships of the sequences identified here to any leader sequences present on IBV mRNAs A and B, but elucidation of their role will probably depend on studies of the mechanism of the viral RNA polymerase responsible for mRNA synthesis.

Acknowledgements

The authors are grateful to Anne Foulds, Ian Foulds and Penny Gatter 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.

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(Manuscript received 14 November 1983)