THE AVIAN CORONAVIRUS MULTIPLICATION STRATEGY

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INTRODUCTION

Infectious bronchitis virus (IBV), the prototype of the coronavirus family, possesses a single-stranded polyadenylated RNA genome. This genome consists of a single polynucleotide chain with an estimated molecular weight of between 6 and 9×10^6 ,^{1,2,3,4}. Since the genome is infectious, i.e., it is of positive polarity,⁵ it must encode both the structural proteins of the virus particle and non-structural polypeptides some or all of which function in the infected cell to replicate the virus genetic material and to transcribe viral mRNA.

Our initial approach in elucidating the coronavirus multiplication strategy has been to examine the nature of the virus-specified RNA species synthesized in primary chicken embryo kidney (CEK) cells infected with IBV. We have described 6 major virus-specified RNAs,⁶. These species comprise RNA F, which is indistinguishable from the viral genome and 5 smaller RNAs, A, B, C, D and E, which have apparent molecular weights of 0.8×10^6 , 0.9×10^6 , 1.3×10^6 , 1.5×10^6 and 2.6×10^6 respectively. Ribonuclease T₁ fingerprinting demonstrated that these subgenomic RNAs constitute a nested set such that the nucleotide sequence of each RNA is contained within all of the larger RNA species. In addition, all of these RNAs are polyadenylated and are therefore likely to be viral mRNAs.

In the present report we show that these subgenomic RNA species share a common 3' end and that all the RNAs extend inward from the 3' end of the genome. We also describe experiments to determine the number and nature of the structural proteins of IBV and to

elucidate the mechanism of synthesis of these proteins in the infected cell.

METHODS

The several methods used in the present work have been described in detail elsewhere,^{1,6,7,8}.

RESULTS AND DISCUSSION

Sequence relationships between the subgenomic RNAs and the viral genome

In order to determine the nucleotide sequence relationship between the 5 subgenomic RNAs and the viral genome we prepared genomic RNA (trace labeled with ³²P-orthophosphate) from purified virus particles, cleaved it with alkali under limiting conditions, fractionated the cleavage products on sucrose gradients and from the gradients selected 7 pools of increasing size from about 8S to about 40S. These pools were then separately chromatographed over oligo(dT)-cellulose to select those fragments which contained 3' terminal sequences and then these pools of 3' terminal fragments were digested with ribonuclease T₁, labeled with polynucleotide kinase and ³²P-γ-ATP and fingerprinted. From these fingerprints a partial 3' to 5' oligonucleotide spot order was deduced. The spots were divided into groups according to which pool first included each spot. Seven groups of oligonucleotides were defined in this way, with each group consisting of the 5' oligonucleotides unique to the corresponding pool. This data is illustrated in Fig. 1 (narrow lines). Pool 1 contained the smallest fragments, so the oligonucleotides in this pool were those closest to the 3' end of the genome and are designated "group 1". The next larger fragments were found in pool 2, which contained the group 1 oligonucleotides as well as a new set. Since pools 1 and 2 differed only at the 5' end, the oligonucleotides specific to pool 2 are located to the 5' side of the group 1 oligonucleotides and these additional oligonucleotides are termed "group 2". The other 5 groups were identified and designated in an exactly analogous fashion. The spot order data were internally consistent in the sense that each fingerprint included all the spots found in fingerprints of smaller pools. When the oligonucleotide fingerprints of each of the 5 IBV subgenomic RNAs A to E were compared with the group spot order it was clear that the intracellular RNAs could be interpolated into the spot order (Fig. 1; heavy lines). Since the spot order pools are a nested series of fragments differing only at their 5' ends, the intracellular RNAs must also form a similar series, with each member of the set sharing a 3' end common to all the intracellular RNA species including the genome. Within the resolution of the data the intracellular RNA species appear to be colinear with the genome, but we cannot rule out the occurrence of small sequence

Fig. 1. Sequence relationship between the 5 intracellular IBV RNAs (drawn in heavy line) and the RNA fragment pools derived from the genome by alkali treatment (drawn in narrow line). Distances from the 5' end of group 8 to the 3' end of group 1 are drawn approximately to scale.

rearrangements such as might result from RNA splicing.

Synthesis of IBV structural proteins

There is general agreement in the literature that the coronaviruses contain 3 major structural polypeptides. These comprise two glycoprotein species with molecular weights of about 100,000 and 30,000 respectively and an internal phosphorylated protein with a molecular weight of about 50,000 (for example, see 9 and review in 3). In addition different laboratories have reported the presence of up to 8 minor polypeptide species. An autoradiograph of the ^{35}S -methionine labeled polypeptides of highly purified IBV is shown in Fig. 2. We routinely observe 8 polypeptides labeled 1 to 8 in Fig. 1. Labeling with ^3H -mannose and ^{32}P -orthophosphate and determination of the apparent molecular weight provides the following information on these polypeptides. Polypeptides 1 and 2 are glycoprotein species with apparent molecular weights of 90,000 and 84,000 respectively (hereafter termed gp90 and gp84). Polypeptide 3 is a phosphoprotein of molecular weight 51,000 (hereafter termed pp51). Polypeptide 4 has a molecular weight of 36,000 (hereafter termed p36). Polypeptide 5 is a glycoprotein with an apparent molecular weight of 31,000 (hereafter termed gp31) and polypeptides 6, 7 and 8 appear to be unmodified polypeptides with molecular weights of 28,000, 23,000 and 14,000 respectively

Fig. 2. Structural polypeptides of IBV. IBV grown in CEK cells in the presence of ^{35}S -methionine was purified by sucrose and renograffin gradient centrifugation, disrupted using SDS, reduced and alkylated and the resultant polypeptides fractionated by polyacrylamide gel electrophoresis. The origin of electrophoresis is at the top of the autoradiograph.

(hereafter termed p28, p23 and p14). Preliminary tryptic peptide mapping of these proteins show that gp90, gp84, pp51, gp31 and p14 are probably all unique polypeptides. By contrast the maps of p36, gp31, p28 and p23 strongly suggest that these four polypeptides all share common amino acid sequences. Of these structural polypeptides only pp51 and gp31 can unequivocally be seen in infected cells. In addition, two other polypeptides, unique to infected cells, are reproducibly seen. These are denoted p165 and p42 in Fig. 3. Cleland mapping clearly shows that p42 is related to pp51. As yet no information is available on the amino acid sequence of p165. A kinetic analysis of the synthesis of these intracellular virus-specified proteins show that they are all synthesized coordinately from the earliest time of detection (~ 3 h post-infection) to the end of the viral multiplication cycle. In an attempt to determine if any of the intracellular proteins are fashioned from high molecular weight precursors we used short pulse and pulse-chase conditions. These experiments, together with experiments using TPCK and amino acid analogues to prevent proteolytic cleavage¹⁰ all failed to identify any high molecular precursor to either pp51 or gp30. From these data we conclude that pp51 and the polypeptide chain of gp30 are primary gene products. However since we have not succeeded in identifying gp90, gp84 or p14 in infected cells their synthesis may involve polyprotein processing. The complex relationship between p36, gp31, p28 and p23 must also be clarified.

Fig. 3. Polypeptides synthesized in cells infected with IBV. CEK cells either mock infected (lane a) or infected with IBV (lane b) were labeled with ³⁵S-methionine for 1/2 h at 7 h post-infection.

Since each of the 5 intracellular virus-specified RNAs contain unique 5' nucleotide sequence information relative to the next smallest RNA species the most likely hypothesis for the translation of these RNAs is that they all function as monocistronic mRNAs and that expression of each one is limited to translation of its unique 5' nucleotide sequence. This hypothesis is consistent not only with our own studies on the intracellular synthesis of pp51 and gp30 but is strongly supported by the observations of Siddell et al.,¹¹ who showed that cells infected with the murine coronavirus JHM contain two mRNA species sedimenting at 17S and 19S and that the former of these is translated in vitro to give a 60,000 dalton protein (the equivalent of pp51) whilst the latter is translated in vitro to give a 23,000 dalton protein which is seen both in infected cells and virions. If the 17S and 19S RNA species correspond to IBV RNAs A and B, which would be congruent with the sedimentation coefficients, the translation results would agree with a non-overlapping translation scheme, with the two RNAs of similar size coding for proteins which differ greatly in size. This concept of a non-overlapping translation scheme would also be in accord with the observation that internal initiation of translation of eukaryotic mRNAs generally does not occur,¹². For example, the internal translation initiation site in the alphavirus genome is cryptic and is only functional in 26S RNA which, like the IBV RNAs, contain nucleotide sequence information from the 3' end of the viral genome,¹³. Indeed the multiplication strategy of IBV and the alphaviruses share many common features. Both are positive-strand RNA-containing enveloped viruses, both appear to replicate extensively if not exclusively in the cytoplasm of the cell and both direct the synthesis of subgenomic RNA(s) whose nucleotide sequence(s) is/are located inward from the 3' end of the genome and which code for the structural proteins of the virus particle. The coronaviruses differ from the alphaviruses however, in that whereas the alphaviruses direct the synthesis of a single subgenomic mRNA which directly encodes all the structural proteins, the coronaviruses direct the synthesis of separate mRNAs for each of their structural proteins. The mechanism by which these several transcriptive events are performed and regulated should prove to be of particular interest.

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