Coronavirus Multiplication: Locations of Genes for Virion Proteins on the Avian Infectious Bronchitis Virus Genome

DAVID F. STERN^{1,2*} AND BARTHOLOMEW M. SEFTON¹

Molecular Biology and Virology Laboratory, The Salk Institute, San Diego, California 92138,¹ and Department of Biology, University of California, San Diego, La Jolla, California 92093²

Received 22 August 1983/Accepted 16 December 1983

Six overlapping viral RNAs are synthesized in cells infected with the avian coronavirus infectious bronchitis virus (IBV). These RNAs contain a 3'-coterminal nested sequence set and were assumed to be viral mRNAs. The seven major IBV virion proteins are all produced by processing of three polypeptides of ca. 23, 51, and 115 kilodaltons. These are the core polypeptides of the small membrane proteins, the nucleocapsid protein, and the 155-kilodalton precursor to the large membrane proteins GP90 and GP84, respectively. To determine which mRNAs specify these polypeptides, we isolated RNA from infected cells and translated it in a messenger-dependent rabbit reticulocyte lysate. Proteins of 23, 51, and 110 kilodaltons were produced. Two-dimensional tryptic peptide mapping demonstrated that these proteins were closely related to the major virion proteins. Fractionation of the RNA before cell-free translation permitted the correlation of messenger activities for synthesis of the proteins with the presence of specific mRNAs. We found that the smallest RNA, RNA A, directs the synthesis of P51, the nucleocapsid protein. RNA C, which contains the sequences of RNA A, directs the synthesis of the small membrane protein P23. RNA E directs the synthesis of the large virion glycoproteins. These results supported a model in which only the unique 5'-terminal domain of each IBV mRNA is active in translation and enabled us to localize genes for virion proteins on the IBV genome.

The coronavirus family was constituted on the basis of the unique morphology of the virion. These viruses attracted interest because the diversity of diseases which they cause makes them good model systems for the study of viral pathogenesis and tissue tropism (reviewed in reference 54). Furthermore, recent studies on the molecular mechanisms of coronavirus replication have demonstrated that the multiplication cycle of coronaviruses differs from that of other animal viruses and is interesting in its own right (reviewed in references 35 and 48).

Coronaviruses are enveloped viruses with exceptionally large continuous RNA genomes which are of positive (messenger) polarity. The genome of avian infectious bronchitis virus (IBV) has an estimated sequence complexity of between 20 and 24 kilobases (kb; 20, 22, 31, 53). We have studied the lytic cycle of this virus to determine how the genetic information is organized and expressed. We describe here the localization of genes for virion proteins on the viral genome.

We have found that IBV-infected cells contain six major viral RNA species (41). These consist of the genome, designated RNA F, and five subgenomic RNAs, designated A, B, C, D, and E in ascending order of size (Table 1). All of these RNAs are likely to function as viral mRNAs, since they are single stranded and polyadenylated. The RNAs were mapped with respect to one another by RNase T₁ oligonucleotide fingerprinting. The results demonstrated that RNAs A, B, C, D, E, and F contain a 3'-coterminal nested sequence set (42). The finding that murine and human coronaviruses specify a similar nested series of RNAs indicated that this transcriptional organization is a characteristic feature of coronaviruses (6, 14, 19, 38, 39, 56).

The fact that coronavirus mRNAs overlap raised the

question of how translationally active sequences are arranged within them. The simplest model is that each mRNA is translated over its entire length. If so, the length of the polypeptide product of a given RNA would be proportional to the size of that mRNA (Table 1). Since there are six presumptive mRNAs but only three possible reading frames, complete translation of the viral RNAs would yield some proteins which necessarily have homologous regions. A second possibility is exemplified by the alphaviruses, which include Semliki Forest virus and Sindbis virus, in which overlapping mRNAs yield unique polypeptides. The single subgenomic alphavirus mRNA is identical in sequence to a 3'-terminal portion of the other viral mRNA, the viral genome (12, 57). However, translation of the genome terminates at a site just upstream from the initiation codon that is active in the subgenomic mRNA (2, 25, 37).

We and others proposed that the coronavirus genes are expressed through a "nonoverlapping" translation scheme that is analogous to translation of alphavirus mRNAs. In this model, only the "unique" 5'-terminal domain that is not contained within smaller mRNAs would be translated, for a terminator of protein synthesis would be present upstream from the translated region of the next smaller mRNA. Thus, the size of the region of each mRNA active in protein synthesis would be approximately equal to the difference in size between it and the next smaller mRNA (Table 1). To test this hypothesis we determined which mRNA specifies each of the major virion proteins.

The major coronavirus virion proteins can be classified into three groups on the basis of size and location in the virion (4, 5, 7, 15, 21, 23, 28, 34, 46, 47, 51, 55; reviewed in references 35 and 48). The first is the nucleocapsid protein, which in the case of IBV is a 51-kilodalton (kd) phosphoprotein. P51 does not undergo posttranslational proteolytic processing (44). The second group is composed of the small, heterogeneous membrane proteins, the P23 family of IBV, and the E1 proteins of murine coronaviruses. The P23 family

* Corresponding author.

† Present address: Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139.

TABLE 1. Coding capacities of IBV mRNAs predicted from two different models

RNA	Length (kb) ^a	Total coding capacity (kd) ^b	Non-overlapping coding capacity (kd) ^c	Size of translation product (kd) ^d
A	2.4	80	80	51
B	2.7	90	10	
M	3.2	110	20	
C	3.9	130	20	23
D	4.5	150	20	
E	7.9	260	110	110
F	23	800	540	

^a The molecular weight of RNA M is based upon unpublished data. The molecular weights of other intracellular RNAs were determined previously (20, 41). The molecular weight of each RNA was divided by 330 to estimate its length in kb.

^b The molecular weight of each RNA was divided by 10 to estimate the size of a polypeptide encoded by the entire RNA.

^c Difference between the coding capacity of each RNA and the coding capacity of the next smaller species.

^d Determined by cell-free translation of fractionated mRNA as described in the text.

consists of P23, GP28, GP31, and GP36. These proteins all contain the same 23-kd core polypeptide and differ only in the number and type of asparagine-linked oligosaccharides that they contain (45). These small coronavirus glycoproteins protrude slightly from the exterior of virions and evidently interact with the nucleocapsid and nucleate assembly of virions on the inner face of the viral membrane (49). This suggests that they span the virion envelope. The third group of coronavirus proteins consists of the large glycoproteins GP84 and GP90 of IBV and E2 of mouse hepatitis virus (MHV). GP90 and GP84 of IBV are structurally unrelated and are produced by posttranslational cleavage of a cell-associated precursor, GP155, at ca. 30 min after synthesis (44). GP90 and GP84 and the homologous E2 glycoproteins of murine coronaviruses comprise the distinctive large surface projections for which coronaviruses are named. These glycoproteins mediate binding of virions to the cell surface and perhaps cause cell fusion late in infection (9, 10).

The seven major IBV virion proteins are thus produced by modification of only three different polypeptides, P23, P51, and the core polypeptide of GP155 (44). Two viral genes, encoding P23 and P51, are monocistronic. However, the gene which encodes GP155 is functionally polycistronic, since this protein is processed to yield two mature proteins. No evidence has been obtained for the production of partly homologous virion polypeptides.

We used the method of cell-free translation to determine which mRNAs specify the major IBV virion proteins. The results enabled us to construct a partial map of the IBV genome. This map is discussed with respect to the organization of the genome of MHV, which is the best-characterized mammalian coronavirus.

MATERIALS AND METHODS

Virus and cells. The Beaudette strain (strain 42) of IBV was propagated in primary chicken embryo kidney (CEK) cells as described previously (40, 41). Cells were incubated at 37 or 38.5°C.

Radiolabeling of intracellular RNA. CEK cultures were washed once with Tris-buffered saline and infected with IBV

at a multiplicity of infection of ca. 10 PFU per cell. After incubation for 90 min, the inoculum was removed. For the experiment shown in Fig. 4, the inoculum was replaced with Dulbecco-Vogt-modified Eagle medium (DMEM) containing 2% calf serum and actinomycin D (a gift from Merck Sharp & Dohme) at a concentration of 1 µg/ml. For the experiment shown in Fig. 5, DMEM containing 2% calf serum was added at the end of the adsorption period, and at 3 h postinfection the medium was replaced with DMEM containing 2% calf serum and actinomycin D.

Cells were labeled with [5,6-³H]uridine (37.6 Ci/mmol; New England Nuclear Corp.) which had been made isotonic by the addition of NaCl to yield a final concentration of 160 mM. For the experiment shown in Fig. 4, label was added at 1 h postinfection to yield a concentration of 0.07 mCi/ml (14 ml per 150-mm dish). Cells were harvested for RNA extraction at 7 h postinfection. For the experiment shown in Fig. 5, [³H]uridine was added at 4.5 h postinfection to yield a concentration of 0.2 mCi/ml (2.4 ml per 60-mm culture) or a concentration of 0.03 mCi/ml (3.1 ml per 100-mm culture). RNA was extracted at 8 h postinfection. The RNA preparation used for the experiment shown in Fig. 2 was the same as that used for the cell-free translation of RNA C which was described previously (40).

Isolation of RNA for cell-free translation. For the experiment depicted in Fig. 5, RNA was isolated from cells by direct extraction of the monolayers with Triton N-101, and the extract was deproteinized with phenol-chloroform in the presence of sodium dodecyl sulfate (SDS; 24). In the experiments depicted in Fig. 2 and 4, cells were scraped into buffer and lysed with Triton N-101, and the nuclei were separated by centrifugation before deproteinization of the cytoplasmic extracts exactly as described before (40). For all in vitro translation experiments, polyadenylated RNA was selected from cytoplasmic RNA preparations by chromatography on oligodeoxythymidylate cellulose as described previously (26, 40).

Analysis of RNA by the Northern blot transfer technique. RNA extracted from IBV-infected cells was denatured in the presence of formaldehyde. The RNA was incubated in 10 mM sodium phosphate (pH 7.4)–50% formamide (MCB Manufacturing Chemists, Inc.)–2.2 M formaldehyde–0.5 mM disodium EDTA at 65°C for 5 min. One-fifth volume of 0.5% SDS–25 mM EDTA–0.025% bromophenol blue–0.1% xylene cyanol FF–25% glycerol was added. Samples were analyzed by electrophoresis in a 1.2% agarose gel which was cast and run in 10 mM sodium phosphate (pH 7.4)–1.1 M formaldehyde (Schleicher & Schuell, Inc., technical information). The RNA was transferred to nitrocellulose paper (50). A *Hae*III restriction fragment of pIBV5 was purified by gel electrophoresis and radiolabeled with ³²P by nick translation. This hybridization probe was incubated with the nitrocellulose filter in a solution containing 50% formamide, 2× Denhardt solution, 0.1% SDS, 200 µg of herring sperm DNA per ml, 5× SSPE (0.9 M NaCl, 5 mM EDTA, 50 mM sodium phosphate, pH 7.7), and 10% dextran sulfate (52) for 48 h at 42°C. The filter was then washed with 0.1× SSPE three times at room temperature.

Analysis of proteins by SDS-polyacrylamide gel electrophoresis. Samples were suspended in electrophoresis sample buffer (5 mM sodium phosphate [pH 7.0], 2% SDS, 0.1 M dithiothreitol, 5% 2-mercaptoethanol, 10% glycerol, 0.4% bromophenol blue), incubated at 100°C for 30 s, and analyzed on discontinuous 15% acrylamide–0.09% bisacrylamide as described previously (32). All gels were 14 cm long and 1 mm thick.

Two-dimensional tryptic peptide mapping. Proteins were eluted from preparative gels after homogenization of the excised gel slices (1). Eluted proteins were precipitated with trichloroacetic acid, oxidized, and digested with tosylsulfonyl phenylalanyl chloromethyl ketone-trypsin (Worthington Diagnostics). Peptides were resolved on 0.1-mm thin-layer cellulose plates (E. M. Reagents) by electrophoresis at pH 4.7 in the first dimension and ascending chromatography in the second dimension (1). The plates were prepared for fluorography (3) and exposed to preflashed film at -70°C .

RESULTS

To facilitate our investigations of IBV, we obtained molecular clones of cDNA copies of the IBV genome (to be described in detail elsewhere [D. F. Stern and B. M. Sefton, unpublished data]). The largest recombinant plasmid, pIBV5, contained a 3.3-kb insert. To determine directly whether pIBV5 contains IBV sequences, we performed a Northern blotting experiment. RNA extracted from IBV-infected cultures was fractionated by agarose gel electrophoresis and transferred to a nitrocellulose filter. A restriction fragment of pIBV5 hybridized to IBV RNAs A, B, C, D, E, and F (Fig. 1) but not to RNA from uninfected cells (data not shown). The limited extent of hybridization to RNA F was probably due simply to poor transfer of this large RNA to the nitrocellulose filter (unpublished data). These data demonstrated that pIBV5 contains IBV sequences. The fact that a small DNA fragment bearing sequences represented near the 3' end of the IBV genome hybridized to all six IBV mRNAs confirmed our previous finding that IBV mRNAs contain common 3' sequences (41, 42).

The experiment depicted in Fig. 1 revealed the existence of a seventh IBV-specific RNA species, designated RNA M, which hybridized to the cDNA probe. RNA M is intermediate in size between RNAs B and C (Table 1) and was sometimes observed as a minor band when viral RNAs were labeled biosynthetically with $^{32}\text{P}_i$ in the presence of actinomycin D (see, for example, reference 43, Fig. 1, lane e). RNA M is likely to be a viral mRNA, but we cannot rule out the possibility that it was derived by nucleolytic cleavage of a larger viral RNA.

Coding assignments for IBV mRNAs. To identify the mRNAs which specify the three major virion polypeptides, polyadenylated RNA purified from infected cells was translated using the messenger-dependent rabbit reticulocyte lysate system (27). IBV-specific translation products were identified by their sizes and by peptide mapping. It was then possible to assign mRNAs to these translation products by fractionation of the RNAs before cell-free translation.

RNA isolated from infected cells (Fig. 2, lane I) yielded many of the same translation products as RNA from mock-infected cells (Fig. 2, lane M). Two obvious exceptions were a protein that comigrated with virion P23 and a series of polypeptides, the smallest of which comigrated with P51. It was likely that these abundant translation products were related to P51 and the P23 family proteins, which are the predominant IBV-specified proteins synthesized in infected cells (44). This conclusion was confirmed by two-dimensional tryptic peptide mapping. The translation product which comigrated with P51 (Fig. 3A) yielded a map similar to that of P51 produced in infected cells (Fig. 3C). A map of a mixture of the peptides confirmed the relationship (Fig. 3D; the variability in migration of the arrowed peptide has been noted before [44]). One of the IBV-specific proteins larger than P51 was also mapped (Fig. 3B). This protein contained

FIG. 1. Northern blot analysis of RNA from IBV-infected cells. RNA was isolated from IBV-infected cells, denatured, and fractionated by agarose gel electrophoresis. The RNA was transferred to nitrocellulose paper, and the filter was incubated with a radiolabeled restriction fragment of pIBV5. The autoradiograph was exposed for 6 days with an intensifying screen (16).

the same tryptic peptides as P51. Every band in this series was also analyzed by the partial proteolytic digestion method of Cleveland (data not shown). All of these proteins were related to P51, and the group was designated the P51 series.

The translation product that comigrated with P23 contained the same major tryptic peptide as GP31 purified from infected cells (Fig. 3E; compare with reference 44, Fig. 4D). This result was confirmed by mapping a mixture of peptides from the *in vitro* translation product and GP31 isolated from infected cells (Fig. 3F) and by partial proteolytic mapping (40, Fig. 6).

mRNAs for P23 and P51. To separate the IBV mRNAs, we employed two different fractionation techniques. The first method was preparative acrylamide gel electrophoresis. RNA was labeled biosynthetically with $^{32}\text{P}_i$ and fractionated on a 2% polyacrylamide gel. IBV RNAs located by autoradiography were eluted from the gel and translated *in vitro*. The translation products of gel-purified RNAs A, B, C, and D are

FIG. 2. Cell-free translation of gel-purified IBV RNAs. RNA was extracted from IBV-infected cells, and individual IBV-specific intracellular RNAs were purified by preparative acrylamide gel electrophoresis (40). The RNA was translated in a messenger-dependent rabbit reticulocyte lysate (1, 27) in the presence of [35 S]methionine (1200 Ci/mmol; ca. 11 mCi/ml; Amersham Corp.), and the products were analyzed by SDS-polyacrylamide gel electrophoresis. The fluorographed gel was exposed to film for 7 days. Lane M, Translation products of RNA from uninfected cells; lane V, translation products of nonfractionated RNA from infected cells; lane A, IBV virion proteins labeled biosynthetically with [35 S]methionine (40); lanes B, C, and D, products of translation of gel-purified IBV RNAs A, B, C, and D, respectively.

shown in Fig. 2. The P51 series was the predominant product of RNA A (lane A). P23 was produced predominantly by translation of RNA C (lane C). RNAs B and D yielded products similar to those obtained from RNAs A and C, respectively (lanes B and D). This can probably be attributed to contamination; no major specific product was produced by translation of RNA B or D.

Fractionation of viral RNA by velocity sedimentation yielded similar results. RNA labeled biosynthetically with [3 H]uridine was isolated from infected cells and sedimented. A portion of each fraction was analyzed by agarose gel

FIG. 3. Tryptic peptide maps of P51 and P23 synthesized in vitro. P23, P51, and a larger member of the P51 series were produced by cell-free translation of RNA from IBV-infected cells in the presence of [35 S]methionine and analyzed by tryptic peptide mapping. Cell-associated forms of P51 and GP31 labeled biosynthetically with [35 S]methionine were mapped for comparison. The proteins were purified by preparative SDS-polyacrylamide gel electrophoresis and digested with trypsin. The resulting peptides were resolved on thin-layer cellulose plates by electrophoresis in the first dimension (from left to right) and by ascending chromatography in the second dimension (from bottom to top). The arrow in Fig. 2, lane A, indicates the member of the P51 series which yielded the map in (B). The map of intracellular P51 (C) and a map of the preparation of GP31 used in the mixture in (F) have been published previously (reference 44, Fig. 4, panels A and E, respectively). Sample activities ranged from ca. 1,000 to 3,000 cpm. Fluorography of the thin-layer plates was for 6 to 15 days. The subscript "t" denotes proteins synthesized by translation in vitro, and "c" indicates proteins from infected cells. (A) P51 synthesized in vitro; (B) larger member of the P51 series synthesized in vitro; (C) P51 synthesized in vivo; (D) mixture of peptides from P51 synthesized in vitro and in vivo; (E) P23 synthesized in vitro; (F) mixture of peptides from P23 synthesized in vitro and GP31 synthesized in vivo.

electrophoresis (Fig. 4, upper panel), and another portion was translated in vitro (Fig. 4, lower panel). The peak of messenger activity for synthesis of the P51 series was in fractions 43 and 44, which contained RNAs A and B. P23 was synthesized by translation of RNA in fractions 39 and 40. The major IBV mRNA in these fractions was RNA C. These data and the results of another gradient fractionation

FIG. 4. In vitro translation of gradient-fractionated RNA. Polyadenylated RNA, labeled biosynthetically with [5,6-³H]uridine, was purified from six IBV-infected CEK cultures in 150-mm dishes. The RNA was fractionated by sedimentation in an 11.6-ml 15 to 30% (wt/vol) sucrose gradient prepared in 50 mM Tris-hydrochloride (pH 7.4)–100 mM LiCl–1 mM EDTA–0.1% SDS. Centrifugation was for 4.5 h at $150,000 \times g$ at 10°C in an SW41 rotor. Fifty-six fractions were collected and numbered consecutively from the bottom of the gradient to the top. RNA was recovered from the fractions by ethanol precipitation in the presence of tRNA carrier, suspended in water, and precipitated again. A portion of the RNA from each fraction was analyzed by agarose gel electrophoresis under non-denaturing conditions (upper panel; 24). Fluorography of the gel was for 7 days. Another portion was translated in vitro, and the products were analyzed by SDS-polyacrylamide gel electrophoresis (lower panel). Sedimentation was from left to right. RNAs from gradient fractions 38 to 47 are shown. RNA in fraction 41 was evidently lost during handling.

FIG. 5. In vitro translation of gradient-purified RNA E. CEK cells in nine 60-mm culture dishes and in four 100-mm culture dishes were infected with IBV and labeled with [5,6-³H]uridine in the presence of actinomycin D. Polyadenylated RNA purified from these cultures was fractionated by sedimentation under the same conditions as described in the legend to Fig. 4. A portion of the RNA was analyzed by agarose gel electrophoresis, and fractions containing RNA E were pooled. RNA recovered from the pooled fractions by ethanol precipitation was analyzed again by agarose gel electrophoresis under non-denaturing conditions (lane a). The cell-free translation products programmed by this RNA preparation in the rabbit reticulocyte lysate were analyzed by SDS-polyacrylamide gel electrophoresis (lane b). Fluorography of the gels was for 7 days (lane a) and 2 h (lane b). Lane a, RNA; lane b, translation products.

experiment (data not shown) supported the conclusion that RNAs A and C specify P51 and P23, respectively.

P110. Digestion of GP155, the precursor to GP90 and GP84, with endoglycosidase H removes most of the carbohydrate and yields a polypeptide of ca. 115 kd (45). We suspected that GP155 was specified by RNA E, the only abundant RNA species which awaited examination. To test this, we purified RNA E by velocity sedimentation. RNA E was by far the most abundant RNA species in this preparation, but small amounts of other viral mRNAs were present as well (Fig. 5, lane a). Translation of this RNA preparation

FIG. 6. Tryptic peptide maps of P110 and GP155. The RNA preparation described in the legend to Fig. 5 was translated in vitro in a reaction containing 50 μ l of reticulocyte lysate and 60 μ Ci of [35 S]methionine. P110 was purified by preparative gel electrophoresis. Tryptic peptides were applied to a thin-layer cellulose plate and separated in two dimensions as described in the legend to Fig. 3. Fluorography was for 60 days. The map of GP155 (labeled biosynthetically with [35 S]methionine) has been published previously (reference 44, Fig. 5, panel C). (A) P110; (B) GP155.

in vitro produced a 110-kd protein, proteins of the P51 series, and some P23 (Fig. 5, lane b). More P51 was produced than P23. This was surprising, since the preparation contained at least as much RNA C as RNA A. One explanation could be that RNA A is more active than RNA C in vitro. Alternatively, P51 messenger activity may have been generated during translation by degradation of larger mRNAs.

To determine whether the 110-kd in vitro product, designated P110, was related to GP155, we compared maps of methionine-labeled tryptic peptides of these two proteins. The peptide maps of P110 (Fig. 6A) and GP155 (Fig. 6B) were nearly identical and demonstrated that these two proteins were closely related. We concluded that RNA E specifies GP155.

DISCUSSION

Primary translation products. Translation in vitro of RNA isolated from IBV-infected cells yielded the polypeptide moieties of the major virion proteins GP90, GP84, P51, GP36, GP31, GP28, and P23. P51 synthesized in vitro was heterogeneous in size. The smallest P51-related polypeptide comigrated with P51 from virions. This polymorphism of P51 produced in vitro could, in principle, result from multiple translational initiation or termination sites.

Indirect evidence suggests that members of the P23 family are transmembrane proteins. Membrane insertion of proteins is generally (but not always) accompanied by cleavage of a signal peptide by an endopeptidase situated on the luminal side of the endoplasmic reticulum (30). Since the rabbit reticulocyte lysate which we used for cell-free translation does not contain microsomal membranes, it has no signal peptidase activity and is incapable of glycosylating proteins on asparagine. If a signal peptide is cleaved from P23 as it is synthesized in vivo, then P23 synthesized in vitro in a lysate without added membranes should be larger, as it would retain the signal peptide. However, P23 synthesized in vitro, virion P23, and intracellular P23 all have exactly the same electrophoretic mobilities (Fig. 2; 40, 44). Thus, it is likely that P23 family proteins do not bear a signal peptide which is removed by signal peptidase.

IBV mRNAs which specify virion proteins. P51, P23, and P110 are specified by mRNAs A, C, and E, respectively. No specific translation products were detected from RNAs B and D. However, the data were not completely convincing because of three technical problems. The first problem was the difficulty in separating the small mRNAs from one another. The second problem was the potential generation of the messenger activities of the smaller RNAs by degradation of larger ones. Since all of the IBV mRNAs contain the sequences of RNA A, nucleolytic degradation could explain the production of some P51 by IBV mRNA preparations which contained little RNA A (Fig. 5). A final problem was the high background of host mRNA activity (Fig. 2). This may have prevented detection of specific translation products of mRNAs B, M, and D. Further confirmation of the conclusions can be obtained by hybrid arrest translation experiments and sequence analysis of the IBV genome.

Map of the IBV genome. Translational initiation sites are generally located near the 5' ends of eucaryotic mRNAs (13). Therefore, coding regions of genes for P51, P23, and P110 almost certainly begin at positions on the genome which correspond to the 5' ends of RNAs A, C, and E, respectively. It is clear from the sizes of the polypeptides specified by RNAs C and E that the entire coding potential of these mRNAs is not exploited (Table 1). The sizes of the primary translation products are, however, compatible with translation of only the unique 5' end of each mRNA (Table 1), and this model will be used as the basis for the discussion which follows. A scale drawing of the IBV genome is depicted in Fig. 7. Genes encoding GP155 (P110), the P23 family proteins (P23), and P51 are indicated. The predicted sizes of the unidentified polypeptides which could arise by nonoverlapping translation are listed in Table 1. The 5' boundaries of regions coding for these polypeptides correspond to the 5' ends of the mRNAs and are marked.

Comparison with the map of MHV. A map of the MHV genome, determined by others using an approach similar to that which we used for IBV (18, 29, 33, 36), is depicted in the lower panel of Fig. 7. The coding assignments for MHV mRNAs are in excellent agreement with the predictions of a nonoverlapping translation scheme.

There is at least one major difference in the transcription of the MHV and IBV genomes. MHV RNA 3 and IBV mRNA E specify polypeptides of similar sizes which are the precursors to the large glycoproteins of the respective viruses. These two mRNAs are therefore homologs. IBV mRNA E is the largest IBV subgenomic mRNA yet detected. However, murine coronaviruses synthesize an abundant subgenomic mRNA which is larger than RNA 3. This species, RNA 2, directs the synthesis of a 30-kd nonstructural protein. IBV and MHV apparently differ in the means by which they express the region upstream from the genes for the large glycoproteins. A transcriptional unit has evidently been gained or lost as IBV and MHV diverged.

The smallest MHV mRNA specifies the nucleocapsid protein, as does the smallest IBV mRNA. Therefore, the map positions of genes which specify both the large glycoproteins and nucleocapsid proteins are similar in IBV and MHV. There is a difference, however, in the positions occupied by the gene for the small coronavirus glycoprotein (E1 or P23). The second-smallest MHV mRNA (mRNA 6), but the fourth-smallest IBV RNA (RNA C), specifies these proteins, respectively. Thus, the gene for E1 of MHV is adjacent to the gene for the nucleocapsid protein. In contrast, the genes for P23 and P51 of IBV are separated by promoters for synthesis of RNAs B and M. This implies that two genes of unknown function separate genes for the nucleocapsid protein and the small membrane protein of IBV.

Blank spaces in the genetic map. The map of known IBV genes encompasses only 25% of the coding potential of the genome. Translation products have been identified for only three of seven putative mRNAs. Since the major virion proteins have been accounted for, candidates for other IBV gene products include only minor components of virions (40) and the nonstructural proteins. The portion of the IBV genome which is not contained within RNA E undoubtedly encodes nonstructural proteins. This 5'-terminal region is larger than the genome of most RNA viruses and has a coding potential of 500 kd. The finding that MHV mutants which are temperature-sensitive for viral RNA synthesis fall into six complementation groups suggests that as many as six independent viral functions may be involved in this process (17).

The coronaviruses, alphaviruses, and tobamoviruses (8, 11) are all positive-stranded viruses which use the same general mechanism of viral gene expression. Each of these virus families employs two or more 3'-coterminal overlapping mRNAs. These subgenomic mRNAs are necessitated by the general inability of eucaryotic ribosomes to initiate translation at internal start codons (13). The use of subgenomic mRNAs makes possible expression of these large viral genomes without the synthesis of correspondingly large primary translation products. An additional advantage to this arrangement is that it permits independent transcriptional regulation of different regions of the viral genome.

Although the general outlines of coronavirus replication are now discernible, our knowledge is still superficial. Further investigation of coronavirus multiplication will be aided enormously by the use of molecular clones of coronavirus sequences. pIBV5 includes sequences derived from 3.3 kb at the 3' end of the IBV genome. Represented among these sequences should be the genes encoding P51 and P23 and presumptive genes demarcated by RNAs B and M. These sequences will also include polymerase recognition sites for synthesis of RNAs A, B, and M and the promoter for synthesis of the negative-stranded template for viral mRNA

FIG. 7. Genetic organization of IBV and MHV. The genomes of IBV and MHV are depicted, with points corresponding to the 5' ends of intracellular RNAs marked. The polypeptides encoded by viral genes are designated by their molecular weights in thousands. The names of the mature forms of the MHV proteins are also indicated, and proteins which are probably unrelated to virion proteins are designated NS. The map of IBV was determined as discussed in the text. The map of MHV is based upon the work of others (18, 29, 33, 34). Upper panel, Map of the IBV genome; lower panel, map of the MHV genome.

synthesis. The use of these clones as a means of determining the nucleotide sequences of these regions, as primers for determining the sequence of the 5' leaders of the mRNAs, as hybridization reagents, and ultimately as substrates for genetic manipulation will lead to a much deeper understanding of coronavirus multiplication.

ACKNOWLEDGMENTS

We are grateful to Tony Hunter for providing copious amounts of the rabbit reticulocyte lysate and for advice and criticism at many stages of this work.

D.F.S. was supported by Public Health Service training grant GM07313 from the National Institutes of Health. This work was supported by Public Health Service grants CA 14195 and CA 17289 from the National Institutes of Health.

LITERATURE CITED

1. Beemon, K., and T. Hunter. 1978. Characterization of Rous sarcoma virus *src* gene products synthesized in vitro. *J. Virol.* **28**:551-566.
2. Bonatti, S., N. Sonenberg, A. J. Shatkin, and R. Cancedda. 1980. Restricted initiation of protein synthesis on the potentially polycistronic Sindbis virus 42S RNA. *J. Biol. Chem.* **255**:11473-11477.
3. Bonner, W. M., and J. D. Stedman. 1978. Efficient fluorography of ^3H and ^{14}C on thin layers. *Anal. Biochem.* **89**:247-256.
4. Cavanagh, D. 1981. Structural polypeptides of coronavirus IBV. *J. Gen. Virol.* **53**:93-103.
5. Cheley, S., and R. Anderson. 1981. Cellular synthesis and modification of murine hepatitis virus polypeptides. *J. Gen. Virol.* **54**:301-311.
6. Cheley, S., R. Anderson, M. J. Cupples, E. C. M. L. Chan, and V. L. Morris. 1981. Intracellular murine hepatitis virus-specific RNAs contain common sequences. *Virology* **112**:596-604.
7. Davies, H. A., R. R. Dourmashkin, and M. R. MacNaughton. 1981. Ribonucleoprotein of avian infectious bronchitis virus. *J. Gen. Virol.* **53**:67-74.
8. Golet, P., and J. Karn. 1982. Tobacco mosaic virus induces the synthesis of a family of 3'-coterminal messenger RNAs and their complements. *J. Mol. Biol.* **154**:541-550.
9. Holmes, K. V., E. W. Doller, and J. N. Behnke. 1981. Analysis of the functions of coronavirus glycoproteins by differential inhibition of synthesis with tunicamycin, p. 133-142. In V. ter Meulen, S. Siddell, and H. Wege (ed.), *Biochemistry and biology of coronaviruses*. Plenum Publishing Corp., New York.
10. Holmes, K. V., E. W. Doller, and L. S. Sturman. 1981. Tunicamycin resistant glycosylation of a coronavirus glycoprotein: demonstration of a novel type of viral glycoprotein. *Virology* **115**:334-344.

11. Hunter, T., R. Jackson, and D. Zimmern. 1983. Multiple proteins and subgenomic mRNAs from a single open reading frame on tobacco mosaic virus RNA. *Nucleic Acids Res.* 11:801-821.
12. Kennedy, S. I. T. 1976. Sequence relationships between the genome and the intracellular RNA species of standard and defective-interfering Semliki Forest virus. *J. Mol. Biol.* 108:491-511.
13. Kozak, M. 1981. Mechanism of mRNA recognition by eukaryotic ribosomes during initiation of protein synthesis. *Curr. Top. Microbiol. Immunol.* 93:81-123.
14. Lai, M. M. C., P. R. Brayton, R. C. Armen, C. D. Patton, C. Pugh, and S. A. Stohman. 1981. Mouse hepatitis virus A59: mRNA structure and genetic localization of the sequence divergence from hepatotropic strain MHV-3. *J. Virol.* 39:823-834.
15. Lanser, J. A., and C. R. Howard. 1980. The polypeptides of infectious bronchitis virus (IBV-41 strain). *J. Gen. Virol.* 46:349-361.
16. Laskey, R. A., and A. D. Mills. 1977. Enhanced autoradiographic detection of ^{32}P and ^{125}I using intensifying screens and hypersensitive film. *FEBS Lett.* 82:314-316.
17. Leibowitz, J. L., J. R. DeVries, and M. V. Haspel. 1982. Genetic analysis of murine hepatitis virus strain JHM. *J. Virol.* 42:1080-1087.
18. Leibowitz, J. L., S. R. Weiss, E. Paavola, and C. W. Bond. 1982. Cell-free translation of murine coronavirus RNA. *J. Virol.* 43:905-913.
19. Leibowitz, J. L., K. C. Wilhelmsen, and C. W. Bond. 1981. The virus-specific intracellular RNA species of two murine coronaviruses: MHV-A59 and MHV-JHM. *Virology* 114:39-51.
20. Lomniczi, B., and I. Kennedy. 1977. Genome of infectious bronchitis virus. *J. Virol.* 24:99-107.
21. Lomniczi, B., and J. Morser. 1981. Polypeptides of infectious bronchitis virus. I. Polypeptides of the virion. *J. Gen. Virol.* 55:155-164.
22. MacNaughton, M. R., and M. H. Madge. 1977. The characterization of the virion RNA of avian infectious bronchitis virus. *FEBS Lett.* 77:311-313.
23. MacNaughton, M. R., M. H. Madge, H. A. Davies, and R. R. Dourmashkin. 1977. Polypeptides of the surface projections and the ribonucleoprotein of avian infectious bronchitis virus. *J. Virol.* 24:821-825.
24. Meinkoth, J., and S. I. T. Kennedy. 1980. Semliki Forest virus persistence in mouse L929 cells. *Virology* 100:141-155.
25. Ou, J.-H., C. M. Rice, L. Dalgarno, E. G. Strauss, and J. H. Strauss. 1982. Sequence studies of several alphavirus genomic RNAs in the region containing the start of the subgenomic RNA. *Proc. Natl. Acad. Sci. U.S.A.* 79:5235-5239.
26. Papkoff, J., T. Hunter, and K. Beemon. 1980. In vitro translation of virion RNA from Moloney murine sarcoma virus. *Virology* 101:91-103.
27. Pelham, H. R. B., and R. J. Jackson. 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur. J. Biochem.* 67:247-256.
28. Rottier, P. J. M., M. C. Horzinek, and B. A. M. van der Zeijst. 1981. Viral protein synthesis in mouse hepatitis virus strain A59-infected cells: effect of tunicamycin. *J. Virol.* 40:350-357.
29. Rottier, P. J. M., W. J. M. Spaan, M. C. Horzinek, and B. A. M. van der Zeijst. 1981. Translation of three mouse hepatitis virus strain A59 subgenomic RNAs in *Xenopus laevis* oocytes. *J. Virol.* 38:20-26.
30. Sabatini, D. D., G. Kreibich, T. Morimoto, and M. Adesnik. 1982. Mechanisms for the incorporation of proteins in membranes and organelles. *J. Cell Biol.* 92:1-22.
31. Schochetman, G., R. H. Stevens, and R. W. Simpson. 1977. Presence of infectious polyadenylated RNA in the coronavirus avian bronchitis virus. *Virology* 77:772-782.
32. Sefton, B. M., K. Beemon, and T. Hunter. 1978. Comparison of the expression of the *src* gene of Rous sarcoma virus in vitro and in vivo. *J. Virol.* 28:957-971.
33. Siddell, S. 1983. Coronavirus JHM: coding assignments of subgenomic mRNAs. *J. Gen. Virol.* 64:113-125.
34. Siddell, S., H. Wege, A. Barthel, and V. ter Meulen. 1981. Coronavirus JHM: intracellular protein synthesis. *J. Gen. Virol.* 53:145-155.
35. Siddell, S., H. Wege, and V. ter Meulen. 1983. The biology of coronaviruses. *J. Gen. Virol.* 64:761-776.
36. Siddell, S. G., H. Wege, A. Barthel, and V. ter Meulen. 1980. Coronavirus JHM: cell-free synthesis of structural protein p60. *J. Virol.* 33:10-17.
37. Simmons, D. T., and J. H. Strauss. 1974. Translation of Sindbis virus 26S RNA and 49S RNA in lysates of rabbit reticulocytes. *J. Mol. Biol.* 86:397-409.
38. Spaan, W. J. M., P. J. M. Rottier, M. C. Horzinek, and B. A. M. van der Zeijst. 1981. Isolation and identification of virus-specific mRNAs in cells infected with mouse hepatitis virus (MHV-A59). *Virology* 108:424-434.
39. Spaan, W. J. M., P. J. M. Rottier, M. C. Horzinek, and B. A. M. van der Zeijst. 1982. Sequence relationships between the genome and the intracellular RNA species 1, 3, 6, and 7 of mouse hepatitis virus strain A59. *J. Virol.* 42:432-439.
40. Stern, D. F., L. Burgess, and B. M. Sefton. 1982. Structural analysis of virion proteins of the avian coronavirus infectious bronchitis virus. *J. Virol.* 42:208-219.
41. Stern, D. F., and S. I. T. Kennedy. 1980. Coronavirus multiplication strategy. I. Identification and characterization of virus-specified RNA. *J. Virol.* 34:665-674.
42. Stern, D. F., and S. I. T. Kennedy. 1980. Coronavirus multiplication strategy. II. Mapping the avian infectious bronchitis virus intracellular RNA species to the genome. *J. Virol.* 36:440-449.
43. Stern, D. F., and B. M. Sefton. 1982. Synthesis of coronavirus mRNAs: kinetics of inactivation of infectious bronchitis virus RNA synthesis by UV light. *J. Virol.* 42:755-759.
44. Stern, D. F., and B. M. Sefton. 1982. Coronavirus proteins: biogenesis of avian infectious bronchitis virus virion proteins. *J. Virol.* 44:794-803.
45. Stern, D. F., and B. M. Sefton. 1982. Coronavirus proteins: structure and function of the oligosaccharides of the avian infectious bronchitis virus glycoproteins. *J. Virol.* 44:804-812.
46. Sturman, L. S. 1977. Characterization of a coronavirus. I. Structural proteins: effects of preparative conditions on the migration of protein in polyacrylamide gels. *Virology* 77:637-649.
47. Sturman, L. S., and K. V. Holmes. 1977. Characterization of a coronavirus. II. Glycoproteins of the viral envelope: tryptic peptide analysis. *Virology* 77:650-660.
48. Sturman, L. S., and K. V. Holmes. 1983. The molecular biology of coronaviruses. *Adv. Virus Res.* 28:35-112.
49. Sturman, L. S., K. V. Holmes, and J. Behnke. 1980. Isolation of coronavirus envelope glycoproteins and interaction with the viral nucleocapsid. *J. Virol.* 33:449-462.
50. Thomas, P. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. U.S.A.* 77:5201-5205.
51. Wade, C. N., and E. G. Westaway. 1981. Structural proteins and glycoproteins of infectious bronchitis virus particles labeled during growth in chick embryo cells. *Intervirology* 15:19-27.
52. Wahl, G. M., M. Stern, and G. R. Stark. 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. *Proc. Natl. Acad. Sci. U.S.A.* 76:3683-3687.
53. Watkins, H., P. Reeve, and D. J. Alexander. 1975. The ribonucleic acid of infectious bronchitis virus. *Arch. Virol.* 47:279-286.
54. Wege, H., S. Siddell, and V. ter Meulen. 1982. The biology and pathogenesis of coronaviruses. *Curr. Top. Microbiol. Immunol.* 99:165-200.
55. Wege, H., H. Wege, K. Nagashima, and V. ter Meulen. 1979. Structural polypeptides of the murine coronavirus JHM. *J. Gen. Virol.* 42:37-47.
56. Weiss, S. R., and J. L. Leibowitz. 1981. Comparison of the RNAs of murine and human coronaviruses, p. 245-259. In V. ter Meulen, S. Siddell, and H. Wege (ed.), *Biochemistry and biology of coronaviruses*. Plenum Publishing Corp., New York.
57. Wengler, G., and A. Wengler. 1976. Localization of the 26S RNA sequence in the viral genome type 42S RNA isolated from SFV-infected cells. *Virology* 73:190-199.