BIOCHEMISTRY OF CORONAVIRUSES 1983

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INTRODUCTION

The first international symposium on coronaviruses was held in 1980. At that time the unique structural features of this virus family were recognised, and much of the discussion centred around evidence for an unusual replication strategy involving a 'nested set' of six subgenomic mRNA's (see Mahy, 1981). Most of the molecular biological studies then reported concerned avian or murine coronaviruses, and these still continue to dominate the field, with little new information available on the bovine, feline or porcine coronaviruses, for example. Major advances in 1983 have resulted from application of the new techniques of nucleic acid cloning and sequencing to the avian and murine genomes, and although only two of the genes (N and E_1) have been analysed so far, the interpretation of experiments to study replication is already on a much firmer basis.

STRUCTURE AND SYNTHESIS OF VIRION PROTEINS

The major spike glycoprotein which forms the characteristic petal-like peplomers of the 'corona' is called the spike or S protein for avian infectious bronchitis virus (IBV) and the E_2 protein for mouse hepatitis viruses. D. Cavanagh (1983 and this volume) has clearly shown by gradient separation of the S protein in the absence of sodium dodecyl sulphate (SDS), that it consists of two polypeptides, S_1 and S_2, of molecular weights 90K and 84K respectively. The molecular weight of the purified spike appears to be 354K ± 17K, and this is consistent with the spike being a tetramer of two S_1 and two S_2 polypeptides. These are apparently not dissociable by β-mercaptoethanol treatment, so are not held together by inter-peptide

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disulphide bonds. Treatment of IBV particles with urea or low concentrations of SDS (0.01%) selectively removes the 90K S_1 polypeptide, leaving S_2 associated with the membrane of the virus.

Evidence that the spike protein of mouse hepatitis virus (strain A59) also consists of two distinct polypeptides was reported by L.S. Sturman (this volume), who terms the subunits, derived from E_2 by trypsin cleavage, as 90A and 90B. He could detect differences in amino-acid composition between the two polypeptides, and also found that the 90A polypeptide contained covalently-bonded palmitic acid in contrast to 90B which did not. Although this is a useful distinguishing marker, the significance of palmitic acid in association with the spike glycoproteins is unclear; as pointed out by R.R. Wagner, two related serotypes of vesicular stomatitis virus (VSV) differ in that the Indiana strain has glycoprotein-associated palmitic acid whereas the New Jersey strain has none. It is tempting to speculate, nevertheless, that the A59 and 90A polypeptide may be membrane associated and so equivalent to the S_2 polypeptide of IBV. Clearly there may also be analogies to the HA_2 portion of the influenza virus haemagglutinin spike, which is much more fully characterised (Wilson et al., 1981).

The preparation of monoclonal or polyclonal antibodies against the murine hepatitis virus E_2 glycoprotein has facilitated further studies of its function, and such antibodies block virus-mediated cell fusion, in addition to neutralising virus infectivity (Collins et al., 1982; Holmes et al., this volume). The application of monoclonal antibodies raised against the bovine enteric coronavirus (Vautherot et al., this volume) has helped to distinguish the 105,000 dalton surface glycoprotein (gp 105) from the other surface glycoprotein component (gp 125). It appears that gp 105 is involved in infectivity and carries haemagglutinating activity in this virus; it may therefore be functionally analogous to the IBV S_1 glycoprotein.

Sturman (this volume) presents evidence that trypsin treatment of A59 virus, which cleaves the 180K E_2 to the 90A and 90B subunit polypeptides, greatly increases the capacity of the virus to cause cell fusion. Whether host cell-dependent protease activation is essential for coronavirus infectivity is still not clear, although there is good evidence in favour of this idea for the bovine enteric coronavirus (Storz et al., 1981). It is possible that virus maturation may be defective in certain cell types which lack proteases of the necessary specificity, and Garwes et al. (this volume) present an interesting example of such a defect. Porcine transmissible gastroenteritis virus grows well in secondary adult pig thyroid cell cultures, but very low virus yields are produced from a pig kidney cell line, LLC-PK1. No defects in virus-specific RNA synthesis were apparent in the two culture systems, and a defect in viral glycoprotein processing seems the most likely explanation for the abortiveness in LLC-PK1 cells.

The second major envelope glycoprotein of coronavirus is known as the E_1 protein in murine viruses, and as the matrix or M protein in IBV. This protein provides the link between the nucleocapsid (N) protein and the envelope, and has been shown to interact directly with RNA of the A59 virus nucleocapsid *in vitro* (Sturman et al., 1980; Holmes et al., this volume). Use of the drug tunicamycin, which in murine virus-infected cells blocks formation of the E_2 but not the E_1 protein, shows that E_1 alone determines the formation of the virus envelope as well as the unusual site of virus budding from the endoplasmic reticulum. It is likely, though not formally proved, that in avian viruses the M protein serves the same function as E_1 in murine viruses. The original experiments with tunicamycin reported at the last meeting (see Mahy, 1981) as well as more formal analysis (Niemann and Klenk, 1981) showed that the carbohydrate moiety of E_1 in murine and in bovine coronaviruses is O-glycosydically linked. By contrast, it is now clear that the M protein of IBV is N-glycosidically linked (Cavanagh, 1983; Stern and Sefton, 1982b). Preliminary nucleotide sequence analysis of the IBV genome by M. Boursnell and T.D.K. Brown has confirmed that the predicted amino-acid sequence of the IBV M protein lacks potential O-glycosylation sites (serine and threonine residues) such as are found at the N-terminus of the murine virus E_1 protein (Armstrong, this volume, Niemann, this volume). Further comparative studies of other coronaviruses would be of interest in this respect, but clearly the existence of this unusual O-linked glycoprotein in the murine and bovine Coronaviridae cannot be used as a hallmark for all members of the family.

O-glycosylation of E_1 appears to be a late event, occurring in the Golgi apparatus, which is not essential for virus maturation. Addition of the glycoprotein transport inhibitor monensin to murine coronavirus-infected cells blocked the glycosylation of E_1 but allowed accumulation of enveloped virions in the endoplasmic reticulum (Niemann et al., 1982). Similar results were obtained in human embryo lung cells with the human coronavirus 229E (Kemp et al., this volume). It can be concluded from these *in vivo* experiments that glycosylation of E_1 is not co-translational, and further evidence for this has been obtained from the elegant *in vitro* study reported by Rottier et al. (this volume). Translation of A59 mRNA in a cell-free system containing dog pancreatic microsomes resulted in synthesis of E_1 protein, the bulk of which was buried in the membrane, only small portions from the N- and C-terminus being expressed in the lumenal and cytoplasmic domains respectively. No evidence for a cleavable N-terminal signal sequence for glycosylation was obtained. It is interesting that the microsomal preparation can be added at any stage during *in vitro* synthesis of the first 150 amino-acids, and the protein will still enter the membrane.

The complete amino-acid sequence of E_1 (predicted from the nucleotide sequence determined by Armstrong et al., this volume) con-

firms the strongly hydrophobic nature of this protein. The M protein of IBV has remarkably similar hydrophobic properties as judged from the available nucleotide sequence data (Boursnell and Brown, this volume). Presumably it is this hydrophobicity which restricts the intracellular migration of E_1 and ultimately results in the occurrence of budding from the endoplasmic reticulum rather than the plasma membrane from where most other enveloped viruses are found to bud.

The third major virion protein of coronaviruses is the nucleoprotein (N) which has a molecular weight of 50-60,000. A nucleotide sequence of the gene encoding this protein in murine A59 virus has been published (Armstrong et al., 1983); two errors in this published sequence were acknowledged and corrected at this meeting (Armstrong et al., this volume). Since the NP gene is located at the extreme 3' end of virion RNA, proximal to the poly(A) tail, this is the easiest gene to reverse transcribe, clone, and sequence, and an additional N gene sequence, that for the JHM strain of MHV, was reported at this meeting (Skinner et al., this volume). The JHM sequence encodes a basic protein of 455 amino-acids which is remarkably similar (94% nucleotide homology in the coding region) to the corrected A59 virus sequence (Armstrong et al., this volume) although two regions of lower homology occur at nucleotide positions 497-569 and 1271-1293 (Skinner et al., this volume). The first 83 nucleotides of the JHM N gene sequence are non-coding, the same as the non-coding nucleotides in A59. As further sequence data become available, comparisons between the highly neurotropic JHM strain and less virulent MHV strains such as A59 will be of considerable interest.

It has been reported that the JHM virion carries an associated protein kinase activity (Siddell et al., 1981a) and this enzyme appears to phosphorylate mainly serine residues in the N protein of murine coronaviruses (Stohlman and Lai, 1979; Siddell et al., 1981a). The role of phosphorylation in the replication cycle is unknown, and for the N protein at least it may be variable, leading to two forms detectable by gel electrophoretic analysis in some systems (Garwes et al., this volume). It has been suggested (Siddell et al., 1981a) that phosphorylation may affect the interaction between N and E_1 protein which has been demonstrated *in vitro* (Sturman et al., 1980) and this possibility would merit further study.

CORONAVIRUS-INDUCED NON-STRUCTURAL PROTEINS

Although a number of non-structural proteins have been detected in murine coronavirus-infected cells, and were described at the last meeting (Siddell et al., 1981b), progress in structural analysis or in assigning functions to these intracellular polypeptides has been disappointing, and they were hardly mentioned at this meeting. At a minimum, the non-structural proteins comprise a 30K - 35K protein, the product of murine coronavirus RNA 2, and a 14K - 17K protein

which has not been precisely mapped, but is encoded in either RNA 4 or RNA 5. However the infectivity of coronavirion RNA means that a further, presumed non-structural, polypeptide awaits identification as the product of RNA 1. Mapping studies indicate that a protein of molecular weight greater than 200K, assumed to have RNA polymerase activity (reviewed by Siddell et al., 1983), is the likely product of this mRNA. Translation of genome RNA in an mRNA-dependent rabbit reticulocyte cell-free system produces three structurally-related proteins of the appropriate size to be the products of this RNA (Leibowitz et al., 1982).

CORONAVIRUS RNA SYNTHESIS

At the time of the last coronavirus meeting the following facts emerged regarding coronavirus intracellular RNA synthesis:-

(i) Coronavirus virion RNA is infectious.

(ii) Up to seven intracellular virus-specific RNA species can be detected in infected cells, one of which is full-length genome-sized RNA, the others being smaller, subgenomic-sized RNAs. All have the same positive polarity as genome RNA.

(iii) All the intracellular RNAs of both avian (Stern and Kennedy, 1980) and murine (Cheley et al., 1981; Lai et al., 1981; Leibowitz et al., 1981) coronaviruses are polyadenylated, have common sequences, and form a 3'-coterminal nested sequence set.

(iv) Synthesis of each of the intracellular RNAs is initiated independently, and not by processing from a large precursor molecule, as revealed by UV transcription mapping experiments (Jacobs et al., 1981; Stern and Sefton, 1982a).

(v) A major area of uncertainty concerned the role of the host cell nucleus in coronavirus RNA synthesis, since it has been suggested (Evans and Simpson, 1980) that IBV replication is α-amanitin- and actinomycin D-sensitive and requires host cell DNA-dependent RNA synthesis.

(vi) Apart from a report concerning porcine coronavirus (Dennis and Brian, 1981), no information on coronavirus-specified RNA polymerase activities was available.

Since the 1980 meeting, the 'nested set' structure of the coronavirus-induced intracellular RNAs has been confirmed (Spaan et al., 1982; Weiss and Leibowitz, 1983) and it has been shown that the subgenomic RNAs, as well as genome RNA, contain 5'-cap structures (Lai

et al., 1982). These RNA molecules were also found to act as individual mRNAs, and to be translated into single proteins of a size corresponding to the coding capacity of the unique 5'-terminal sequences not present in the next smallest RNA (Rottier et al., 1981; Leibowitz et al., 1982; Siddell, 1983).

Summarising the last meeting, I suggested that although the mRNAs appeared to be synthesised independently, the existence of a short 5'-terminal sequence common to all the RNAs and derived by a splicing or polymerase jumping mechanism could not be excluded (Mahy, 1981). Surprisingly, this has proved to be the case.

Lai et al. (1982a) first showed that the 5'-termini of most of the MHV-A59 virus-specific RNAs induced in infected L2 cells contained a common tetranucleotide sequence, 5'-cap-N-UAAG, identical to the 5'-terminal genome RNA sequence. In addition, T_1 oligonucleotide mapping of MHV-A59 mRNAs revealed one oligonucleotide, No.10, which mapped at the 5' terminus of mRNAs 2, 3, 5, 6 and 7 but only occurred once within the genome. This leads inescapably to the conclusion that a sequence containing oligonucleotide 10 is somehow translocated from its position in genome RNA onto the 5' terminus of each mRNA, and so must constitute a leader sequence. A consequence of such a translocation would be the formation of new T_1 oligonucleotides at the junction of the leader and the body sequence of each mRNA, and such candidate oligonucleotides found in mRNA but not in genome RNA, are oligonucleotides 19 and 19a (Lai et al., 1982a; Lai et al., this volume). Similar results were reported by two other groups (Leibowitz et al., 1981; Spaan et al., 1982).

The size of oligonucleotide 10 was determined to be 23 nucleotides (Lai et al., 1982a), and oligonucleotides 19 or 19a apparently have 22 nucleotides (Lai et al., this volume). The total length of the postulated leader sequence is not known, but can be estimated as at least 40 nucleotides. These elegant and painstaking oligonucleotide mapping studies by Lai et al. (this volume) have been the stimulus to two alternative approaches which have confirmed the existence of leader RNAs on coronavirus mRNAs.

In the first of these approaches, single-stranded cDNA copied from the smallest subgenomic mRNA (7), was hybridised with genome RNA or subgenomic mRNAs then examined in the electron microscope after cytochrome spreading. The bulk of the cDNA (approximately 2000 nucleotides) hybridised to the 3' terminal region of genome RNA, but large loops of RNA were also seen, consistent with a short region (around 50 nucleotides) of homology between the cDNA and the 5' terminus of genome RNA (Spaan et al., this volume). Hybridisation of the mRNA 7 cDNA to mRNA 6 also resulted in the formation of looped hybrid structures.

The second approach has been direct sequence analysis of mRNA7 and the corresponding region of the genome. This confirmed the existence of a 'fusion-sequence' since the nucleotide sequence of the 5' region of mRNA 7 of A59 virus could be seen to diverge from the corresponding region of the genome upstream from the N gene iniatiation codon (Spaan et al., this volume). The 5'-terminus of mRNA 7, but not of the genome, contains sequences which correspond to oligonucleotides 10 and 19 as reported by Lai et al. (1982a). The fusion sequence of mRNA 7 was shown to be within oligonucleotide 19.

The RNA synthetic mechanism which generates these fused sequences is of considerable interest. Lai et al. (1982b) reported that the template for mRNA synthesis is a single, genome-length, negative-stranded RNA molecule, and no evidence for multiple negative-stranded RNAs has been obtained. Replication of murine coronaviruses, at least, seems to be confined to the cytoplasm since enucleated cells support A59 or JHM virus growth and inhibitors of cell DNA transcription such as α-amanitin or actinomycin D have no effect on virus yield (Brayton et al., 1981; Wilhelmsen et al., 1981; Mahy et al., 1983). (There remains some doubt concerning the role of the cell nucleus in avian coronavirus replication). From these data, the possibility that murine coronavirus mRNAs acquire leader sequences by splicing in the cell nucleus can be ruled out. Processing of a large precursor RNA in the cytoplasm is also excluded by the transcriptional mapping data which show that the UV target size of each mRNA corresponds to its physical size (Jacobs et al., 1981; Stern and Sefton 1982a). Thus each mRNA must be individually transcribed at its own initiation point on the negative strand template.

Two possible mechanisms by which the leader sequences could become fused to the mRNA body sequences during transcription were considered at the meeting. The first would involve the bringing together of non-contiguous sequences on the negative strand RNA template due to secondary structure alterations which lead to looping or folding of the molecule. The polymerase would then read the leader sequence and jump across a postulated gap in the template, joining the leader to mRNA body sequences in the process. Such a jumping mechanism, which involves the influence of ribonucleoprotein structure in bringing together non-contiguous sequences, is thought to be involved in the generation of defective interfering RNAs during influenza virus transcription (Fields and Winter, 1982; Jennings et al., 1983). The second mechanism involves reinitiation of the transcription process at a point near the start of each gene on the template; the leader RNA sequence would remain attached to the polymerase after its own synthesis, and serve to prime transcription at six different regions along the template. Although data is not yet available for murine coronavirus, it is interesting that Boursnell and Brown (this volume) have detected two regions of homology on the IBV genome which might be the primer attachment points.

Lai et al. (this volume) present further evidence in favour of the second mechanism. Only one size (full-length) of double-stranded replicative form (RF) molecule was found in infected cells after ribonuclease treatment. If the first mechanism were correct, multiple RF's would be expected to be generated by this procedure. Furthermore, they were able to isolate replicative intermediate (RI) structures containing single-stranded tails by precipitation with 2M lithium chloride. Only one species of RI, migrating faster than genome RNA, was found by gel electrophoresis. This species was 40-60% resistant to ribonuclease, and had a structure which suggested the existence of six single-stranded tails on each full-length template RNA. Evidence was also obtained that poly(A) is added to the mRNAs during synthesis and not post-transcriptionally.

The nature of the RNA-dependent RNA polymerase responsible for these events is still unclear. Since the report by Dennis and Brian (1981) on the induction of RNA polymerase activity by porcine coronavirus, two reports have appeared describing a similar enzyme activity in murine coronavirus-infected cells (Brayton et al., 1982; Mahy et al., 1983). No evidence for the existence of such an enzyme in cells infected with other coronaviruses, such as IBV, has been presented. Brayton et al. (1982) could distinguish two polymerase activities, one early (one hour post-infection) and the other late (six hours post-infection). The latter activity corresponds to the one detected by Mahy et al. (1983). The current hypothesis, presented by Lai et al. (this volume) would favour separate polymerase activities for the synthesis of negative and positive strands, and since the early polymerase activity declines rapidly, the negative-stranded RNA template would need to be rather stable. Considerable further work on the products of these enzymes is needed to establish the events involved in the unique RNA synthetic mechanism induced by coronavirus infection.

ACKNOWLEDGMENT

I wish to thank Mrs. Mary Wright for her excellent typing of the manuscript.

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