INTRACELLULAR PROTEIN SYNTHESIS AND THE IN VITRO TRANSLATION OF CORONAVIRUS JHM mRNA

Stuart Siddell, Helmut Wege, Andrea Barthel and Volker ter Meulen

Institute of Virology, University of Würzburg, FRG

1. Introduction

The genome of the murine coronavirus JHM is a single-stranded, non-segmented RNA with a molecular weight of 5.4 - 6.7 x 10^6. The genomic RNA is infectious and at least one third is polyadenylated (Lai and Stohlman, 1978; Wege et al., 1978). The virion contains six proteins, four of which are glycosylated (Wege et al., 1979; Siddell et al., 1981b), including high and low molecular weight glycoproteins and a phosphorylated nucleocapsid protein of 60,000 molecular weight (Stohlman and Lai, 1979; Siddell et al., 1981a). Associated with purified virions is a protein kinase activity which specifically phosphorylates pp60 in vitro (Siddell et al., 1981a).

Information on the replication of JHMV is relatively incomplete. The synthesis and processing of JHMV proteins in infected cells, the genesis of genomic and subgenomic viral RNA, and the translation products of viral mRNA are all areas currently under investigation (Anderson et al., 1979; Bond et al., 1979; Siddell et al., 1980, 1981b; Wege et al., 1981). In this communication we report on the synthesis of polypeptides in JHMV infected cells and the in vitro translation of coronaviral mRNA in cell-free systems.

V. ter Meulen et al. (eds.), *Biochemistry and Biology of Coronaviruses*

2. Materials and Methods

The following methods used here have been described previously. JHM virus and virus stocks were obtained and propagated on Sac (-) cells and virions were labelled with 35 S methionine and purified as described (Siddell et al., 1980). Cells were infected, labelled with 35 S methionine and cell or cytoplasmic lysates were prepared as described (Siddell et al., 1981b). The preparation of anti JHM serum in rabbits and immunoprecipitation procedures have been described (Siddell 1980; 1981b). The procedures for the preparation of cytoplasmic polyadenylated RNA and polysomal RNA from infected cells, sucrose-formamide gradient centrifugation and the translation of RNA in nuclease treated rabbit reticulocyte lysates or L cell lysates is described (Siddell et al., 1980). Samples were electrophoresed on discontinuous 15 % SDS/polyacrylamide gels (Laemmli, 1970) and the procedures for staining, drying and autoradiography of gels is described (Smith et al., 1974). Tunicamycin was obtained as a gift from Dr. R. Hamill, Lilly Research Laboratories, Indianapolis.

3. Results

Under our conditions cells infected with JHMV at a MOI of 5 TCID 50/cell start to release infectious virus 4 to 6 hours after infection. The cytopathic effect of JHMV is characterized by the formation of syncytia leading to a totally fused monolayer which is evident by approximately 10 hours after infection. After 12 hours of infection the cell monolayer starts to detach from the petri dish.

3.1. Virion proteins

The 35 S-methionine-containing proteins of purified JHMV are shown in Fig. 1. The virion is comprised of six major proteins which are designated by their molecular weight and whether or not they are glycosylated (Wege et al., 1979; Siddell et al., 1981b).

3.2. Intracellular protein synthesis

Fig. 2 shows the pulse labelling of cells for 15 minutes with 35 S-methionine at different times after infection. At late times in infected cells it is possible to detect the synthesis of three major (150 K, 60 K and 23 K) and three minor (65 K, 30 K and 14 K) polypeptides. The synthesis of the 30 K species is sometimes difficult to detect and the 150 K polypeptide is reproducibly resolved as a doublet.

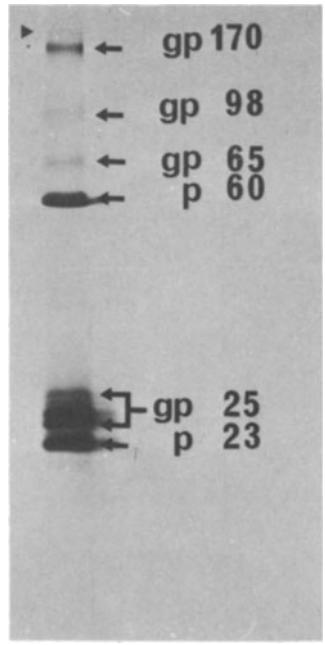

Fig.1: Structural proteins of JHMV. JHM virions were labelled with 35 S-methionine and purified as described in Siddell et al. (1980). Virus was mixed with an equal volume of electrophoresis sample buffer and heated to 37 °C for 2 minutes before electrophoresis.

To investigate any relationships between these intracellular polypeptides and the virion proteins, a 35 S-methionine pulse labelled cytoplasmic lysate was immunoprecipitated with serum directed against purified virions and the immunoprecipitate co-electrophoresed with labelled virus (Fig. 3, tracks V and pulse). This experiment shows that the three major intracellular species, 150 K, 60 K and 23 K and one minor species, 65 K, are specificlly immunoprecipitated (immunoprecipitation of infected lysates with control serum were negative).

These species are therefore related to virion proteins. The 65 K, 60 K and 23 K species comigrate with the virion proteins gp 65, p 60 and p 23. The 150 K species does not comigrate with a virion protein. The intracellular species of 30 K and 14 K which are not precipitated are assumed to be polypeptides which are not

present in purified virions. The minor band migrating slightly faster than the 60 K species is generated during the immunoprecipitation procedure and is presumed to be a degredation product, most probably of nucleocapsid protein.

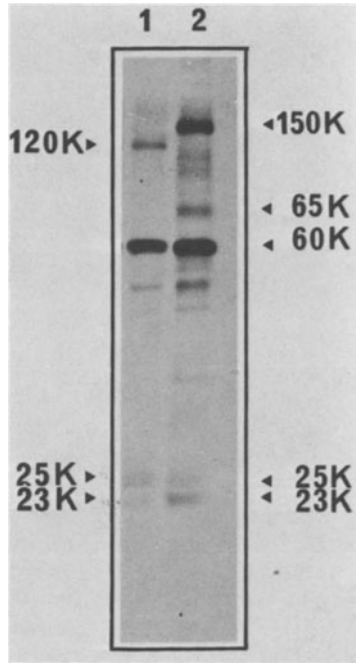

Fig.2: Polypeptides synthesized in JHMV-infected cells. Sac (-) cells were infected with JHMV and labelled for 15 minutes at the times indicated in medium containing 10 μCi/ml 35 S-methionine, and lysates were prepared and electrophoresed as described in Siddell et al., 1981b. M. Mock-infected, MW. Molecular Weight markers.

Fig. 3 also shows the immunoprecipitation with the same antiserum of cytoplasmic lysates pulsed with 35 S-methionine and then chased in the presence of an excess of methionine for various periods of time. During the chase period, the 150 K and 23 K species are processed and two new immunoprecipitable species of 98 K and 25 K appear. Also a small amount of material of 170 K and material which remains at the origin of the gel appear during the chase period. As we have shown (Siddell et al., 1981b) that boiling purified virions in

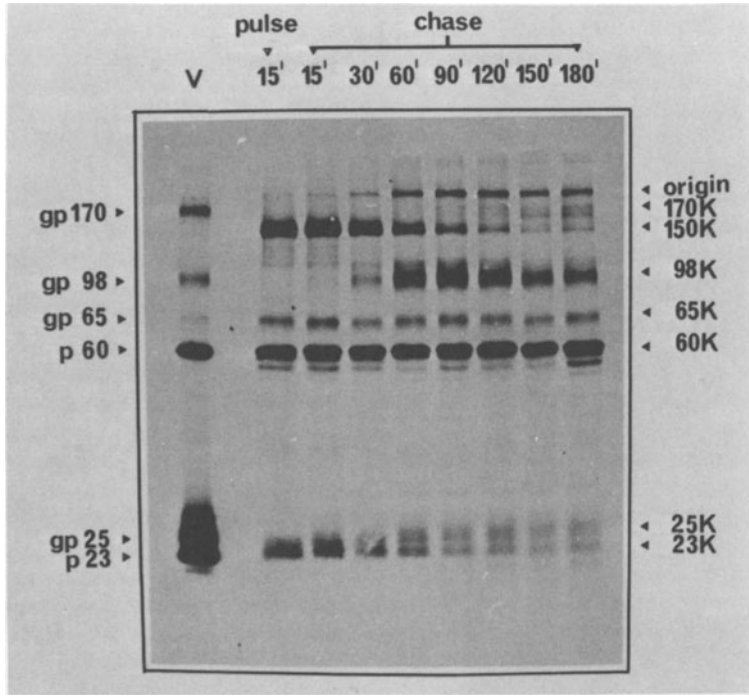

Fig.3: Pulse-chase labelling of JHMV-infected cells and immunoprecipitation of cytoplasmic lysates with anti-JHM serum. Sac (-) cells were infected with JHMV, pulse-labelled 9 hours after infection in medium containing 10 µCi/ml 35 S-methionine and chased with medium containing unlabelled methionine as described in Siddell et al., 1981b. Cytoplasmic lysates were prepared and aliquots immunoprecipitated with anti-JHM serum and electrophoresed. Purified virus (V) was prepared as described in Fig. 1.

electrophoresis sample buffer caused the disappearance of gp 170 and the appearance of material at the gel origin, we repeated this experiment without boiling the immunoprecipitated proteins. Under these conditions no radioactivity appears at the gel origin during the chase period but instead the intracellular species of 170 K is enhanced (data not shown). The intracellular species of 170 K, 98 K and 25 K which arise during the chase period comigrate with virion proteins gp 170, gp 98 and gp 25.

The effect of tunicamycin on the synthesis of JHMV proteins is illustrated in Fig. 4. When infected cells are pulse labelled for 1 hour with 35 S-methionine

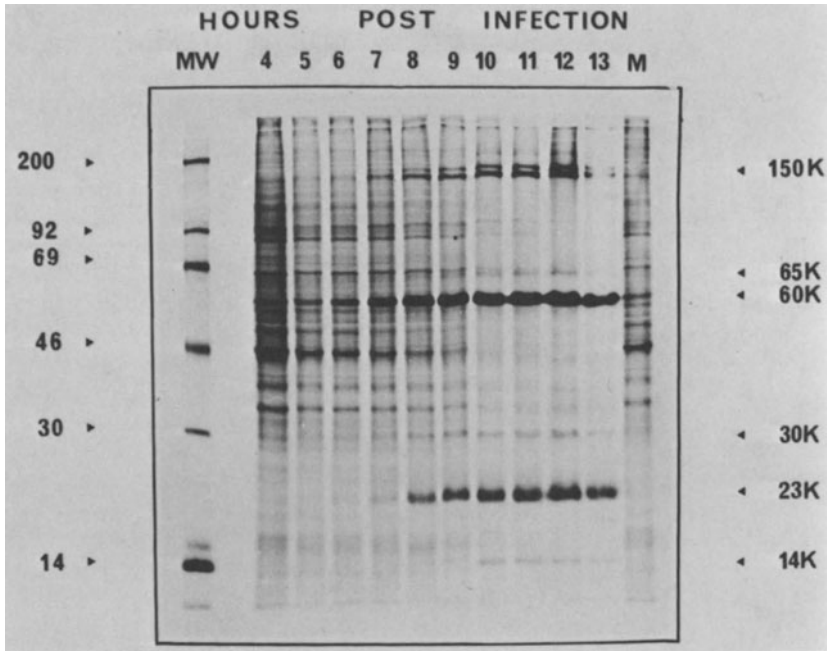

Fig.4: The effect of tunicamycin on JHMV polypeptide synthesis. Sac (-) cells were infected with JHMV and labelled 9 hours after infection for 1 hour in the presence (1) or absence (2) of tunicamycin (1 µg/ml). Cells labelled in the presence of tunicamycin were pretreated for 2 hours with the drug, at the same concentration. Cytoplasmic lysates were prepared, immunoprecipitated with anti JHM serum and the precipitates electrophoresed as described in Siddell et al., 1981b.

and a cytoplasmic lysate immunoprecipitated with anti-JHM serum, polypeptides of 150 K, 98 K, 65 K, 60 K, 25 K and 23 K are detected (track 2). In cells incubated with medium containing 1 µg/ml of tunicamycin during and for 2 hours previous to the labelling period, a different pattern is seen (track 1). The 150 K, 98 K and 65 K species are no longer detected and a new species of 120 K is found. The synthesis of the 60 K, 25 K and 23 K proteins is not affected by tunicamycin treatment.

3.3. In vitro translation

In cells infected with JHMV pulse labelling with 3 H uridine in the presence of actinomycin D reveals the synthesis of 9 RNAs. These range in size from 0.62×10^6 to 6.67×10^6, the size of JHMV genomic RNA. All these

RNAs are single stranded and at least a proportion of each is polyadenylated (Wege et al., 1981). To investigate whether these species function as mRNA we have isolated poly-A- containing RNA from infected cells, translated it in vitro and compared the products with JHM polypeptides by electrophoresis and immunoprecipitation. Furthermore we have size fractionated the translational activities in order to

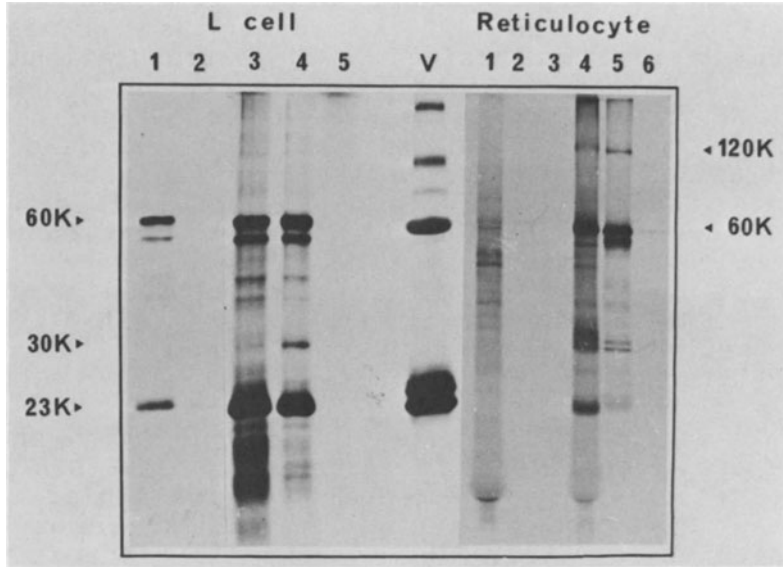

Fig.5: Polyacrylamide gel analysis of proteins made in vitro in response to cytoplasmic poly A RNA or polysomal RNA from JHMV infected cells. Cytoplasmic poly A RNA and polysomal RNA was isolated, translated in vitro, electrophoresed directly or immunoprecipitated with anti JHM serum and electrophoresed as described in Siddell et al., 1980. Purified virus (V) was prepared as described in Fig. 1. L cell translates, (5) no added RNA, (4) cytoplasmic poly A RNA, (3) polysomal RNA. Immunoprecipitation of (4) with preimmune serum (2) or with anti-JHM serum (1). Reticulocyte translates, (1) cytoplasmic poly A RNA from uninfected cells. Immunoprecipitation of (1) with anti JHM serum (2) or pre immune serum (3). (4) cytoplasmic poly A RNA from JHMV infected cells. Immunoprecipitation of (4) with anti JHM serum (5) or preimmune serum (6).

correlate individual mRNAs with their translation products.

Fig. 5 shows the translation of cytoplasmic poly A RNA from infected cells in L cell and rabbit reticulocyte lysates (tracks L4 and R4). Amongst the major products are polypeptides with molecular weights of 23 K, 60 K and 120 K which are specifically immunoprecipitable with anti-JHM serum (tracks L1 and 2 and R5 and 6). The 23 K and 60 K products coelectrophorese with virion p23 and p60 respectively (track V) whilst the 120 K product has no virion counterpart. Also synthesized in these translations are a major 30 K product (seen most clearly in L4) and a minor 14 K product (see also Fig. 6 A or C) neither of which is immunoprecipitable with anti-JHM serum. We have also translated RNA released from infected cell polysomes (Siddell et al., 1980). The result is shown in track L3 and is essentially identical to that obtained with total cytoplasmic poly A RNA, except that the 30 K product is made, if at all, in much reduced amounts. Polysomal RNA and total cytoplasmic poly A RNA directed the synthesis of approximately equal amounts of the 60 K and 23 K products in the experiment .

To establish the size of the RNAs which encode the viral polypeptides we sedimented poly A RNA from infected cells on sucrose-formamide gradients, recovered RNA from fractions of the gradients and translated it in vitro. Fig. 6 shows the products made in the L cell system (A and C) and the reticulocyte lysate (B) in response to unfractionated RNA (total) and RNA sedimenting between about 12S and 45S (A and B) or 17S to 22S (C). The RNA encoding the 60 K product sediments at about 17S, that encoding the 23 K product at 19S and that encoding the 14 K product at 21S (C). The RNAs encoding the 120 K and 30 K products sediment faster, at about 27 S and 29 S respectively (B and A).

4. Discussion

The protein composition of JHMV described here is in agreement with earlier reports (Wege et al., 1979). It also conforms to the pattern which appears to be emerging as characteristic of coronaviruses, namely 5 to 8 proteins, including high and low molecular weight glycoproteins and a 50 to 60 K phosphorylated nucleocapsid protein (Sturman et al., 1977; Sturman and Holmes, 1977; Bond et al., 1979; Siddell et al., 1981 a,b; Cavanagh, 1981; Garwes and Pocock, 1975; Pocock and Garwes, 1977; MacNaughton, 1980).

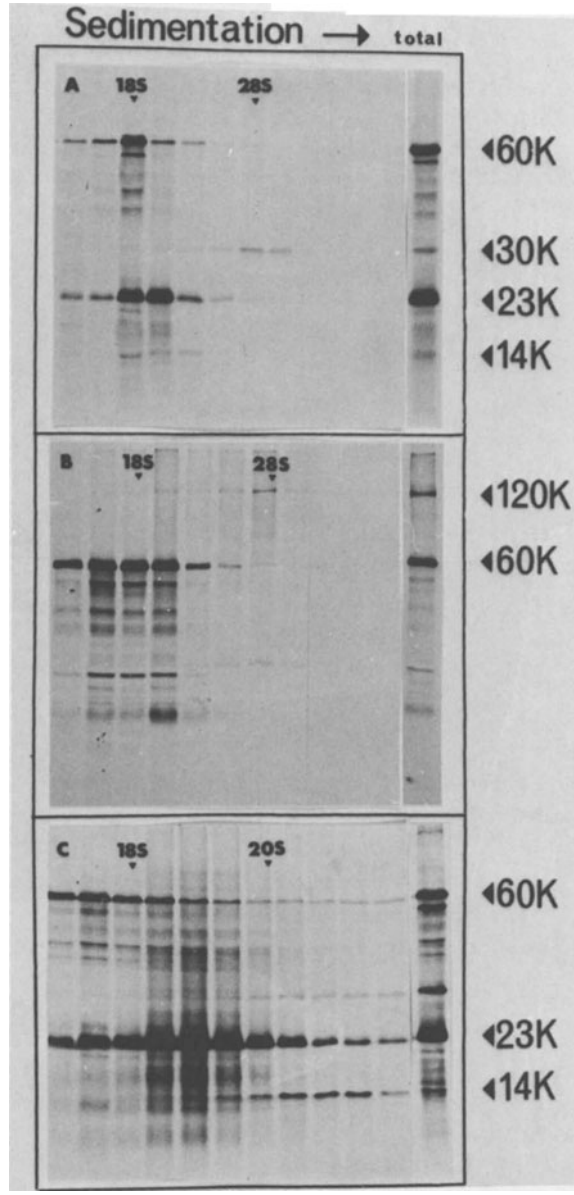

Fig 6: In vitro translation of fractioned poly A RNA from JHMV infected cells. Poly A RNA was sedimented on sucrose formamide gradients, recovered from fractions of the gradients and translated in a L cell lysate (A and C) or a reticulocyte lysate (B). The products were electrophoresed directly (A and C) or immunoprecipitated with anti JHM serum and electrophoresed (B).

In infected cells four polypeptides are synthesized which can be identified by immunoprecipitation as related to virion proteins. Pulse chase labelling, immunoprecipitation and coelectrophoresis with virion proteins suggest that the four polypeptides give rise to all virion proteins. The pathways of processing have yet to be rigorously established but our interpretation of these results is illustrated in Fig. 7.

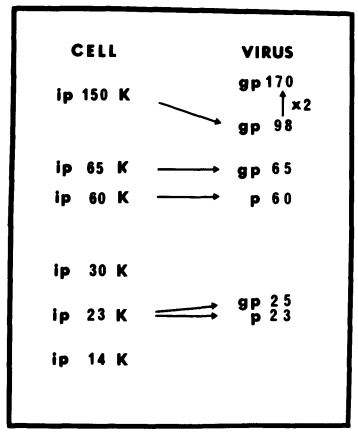

Fig.7: A tentative model for the synthesis and processing of coronavirus JHM proteins.

The intracellular 65 K, 60 K and 23 K polypeptides are incorporated into virions as gp 65, pp60 and p23. The intracellular 150 K polypeptide is processed to a 63 K species which is not incorporated into virions (unpublished data) and a 98 K species and its 170 K dimer, both of which are incorporated into virions, as gp 98 and gp 170 respectively. In the low molecular weight region the intracellular 23 K species is glycosylated to produce the 25 K polypeptide which is incorporated into

the virus as gp 25. Tunicamycin inhibits the glycosylation (and consequent processing) of the 150 K species. The 120 K species synthesized in tunicamycin treated cells is presumed to be the polypeptide core of the 150 K protein. The glycosylation of the intracellular 65 K species also appears to be inhibited by tunicamycin but no core protein has yet been found. In contrast tunicamycin does not prevent the glycosylation of the 23 K species to yield gp 25. These conclusions, although tentative, are supported by the electrophoresis of intracellular polypeptides on two-dimensional (NEPHGE or IEF) PAGE (Siddell et al., 1981b) and in vitro translation studies (Siddell et al., 1980). Our tryptic peptide mapping studies which are in progress are also consistent with this interpretation of the data, and additionally confirm that the 30 K and 14 K species identified as putative non-structural proteins are unrelated to virion polypeptides (unpublished data).

The translation of RNA from JHMV infected cells provided some of the first evidence for the existence of multiple subgenomic coronavirus mRNAs (Siddell et al., 1980). Analysis of RNA from murine and avian coronavirus infected cells indicates there to be the genomic sized RNA and possibly up to 8 subgenomic RNAs which could function as mRNAs (Stern and Kennedy, 1980a,b, Wege et al., 1981, van der Zeijst, 1981). We have attempted to demonstrate directly the mRNA function of these RNAs by in vitro translation. Our results suggest that amongst the translation products of total cytoplasmic poly A RNA are polypeptides which give rise to all structural proteins of JHMV. The only exception is gp 65, an in vitro counterpart for which we have not yet identified. The in vitro synthesis of precursors to pp60 and p23 (and therefore as postulated gp 25) has been confirmed by electrophoresis, immunoprecipitation and tryptic peptide mapping (Siddell et al. 1980; unpublished data). In vivo experiments with tunicamycin suggest that the non-glycosylated form of the intracellular 150 K species has a molecular weight of 120 K. This corresponds to an in vitro product immunoprecipitable with anti JHM serum and we conclude this species to be related to the intracellular 150 K protein and as postulated gp 98 and gp 170 in the virion.

Additionally, we have shown that the mRNAs encoding the virion polypeptides are subgenomic, as are those encoding the 30 K and 14 K putative non-structural proteins. This conclusion is based on the sedimentation

of mRNA activities in sucrose formamide gradients. To correlate these activities with the subgenomic RNA species identified in infected cells we have analysed on gels 32 P labelled RNA after recovery from gradients. The expected relationship was found in that larger RNA sedimented faster, i.e. RNA 1, 45 - 50S; RNA 2, 30S; RNA 3, 28S; RNA4 and 5, 21 - 22S; RNA 6, 19S and RNA 7, 17S, although the sedimentation of the larger RNAs is slower than may have been expected.

Taken together the data allows us tentatively to identify the majority of subgenomic JHM mRNAs and their translation products which is shown in Fig. 8.

RNA	SIZE ($x10^{-6}$)	SIZE (S)	TRANSLATION PRODUCT
1	6.67	45-50	not identified
a	4.7		not identified
2	3.42	30	30 K ▸ ns
3	2.76	28	120 K ▸ gp170/98
b,4,5	1.19-1.5	21-22	14 K ▸ ns
6	0.93	19	23 K ▸ p23/gp25
7	0.62	17	60 K ▸ pp60

Fig.8: Tentative coding assignments for coronavirus JHM subgenomic mRNAs.

At the moment we have detected only one translational activity sedimenting at about 21S, i.e. that encoding the 14 K protein. As two major and one minor RNA species sediment at about this value we cannot conclude which of these three RNAs encodes this protein or if the remaining two RNAs act as mRNA. We have also found no translational activity at the sedimentation value of the minor RNA a, nor have we identified any translation product for the intracellular genomic sized RNA 1. As the translation of the mRNAs encoding all major virion proteins has been demonstrated it seems most likely that RNA 1, if functional, encodes a non-structural

protein(s).

The size of the murine coronavirus subgenomic mRNAs and their translation products, together with the known sequence arrangement of coronavirus IBV intracellular RNAs (Stern and Kennedy, 1980 a, b) is consistent with a replication strategy which involves multiple subgenomic mRNAs. Translation studies show that each mRNA functions monocistronically, although additional coding sequences are present in all but the smallest mRNA. This strategy appears to be particularly flexible in that it allows firstly for the control of viral protein synthesis at both transcriptional and translational levels and secondly the possibility to spatially separate the translation and post translational modification of different viral polypeptides into different cellular compartments.

Acknowledgement

We thank Helga Kriesinger for typing the manuscript. This research was supported by Deutsche Forschungsgemeinschaft Me 270/18.

References

Anderson, R., Cheley, S. and Haworth-Hatherell, H., (1979). Comparison of polypeptides of two strains of murine hepatitis virus. Virology 97, 492-494.

Bond, C.W., Leibowitz, J.L., and Robb, J.A., (1979). Pathogenetic murine coronaviruses. II. Characterization of virus specific proteins of murine coronaviruses JHMV and A59V. Virology 94, 371-384.

Cavanagh, D. (1981). Structural polypeptides of coronavirus IBV. Journal of General Virology, in the press.

Garwes, D.J. and Pocock, D.H. (1975). The polypeptide structure of transmissible gastroenteritis virus. Journal of General Virology 29, 25-34.

Laemmli, U.K., (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227: 680-685.

Lai, M.M.C. and Stohlman, S.A. (1978). RNA of mouse hepatitis virus. Journal of Virology 26:236-248.

Macnaughton, M.R. (1980). The polypeptides of human and mouse coronaviruses. Archives of Virology 63, 75-80.

Pocock, D.J. and Garwes, D.H. (1977). The polypeptides of haemagglutinating encephalomyelitis virus and isolated subviral particles. Journal of Virology 37, 487-499.

Siddell, S.G., Wege H., Barthel, A. and ter Meulen, V. (1980). Coronavirus JHM: Cell-free Synthesis of Structural protein p60. Journal of Virology 33, 10-17.

Siddell, S.G., Barthel, A. and ter Meulen, V. (1981a). Coronavirus JHM. A virion-associated protein kinase Journal of General Virology, in the press.

Siddell, S.G., Wege, H., Barthel, A. and ter Meulen, V. (1981b). Coronavirus JHM. Intracellular protein synthesis. Journal of General Virology, in the press.

Smith, A.E., Wheeler, T., Glanville, N. and Kääriäinen, L. (1974). Translation of Semliki Forest virus 42S RNA in a mouse cell-free system to give virus coat proteins. European Journal of Biochemistry 49, 101-110.

Spaan, W.J.M., Rottier, P.J.M., Horzinek, M.C. and van der Zeijst, B.A.M. (1981). Isolation and identification of virus specific mRNA's in cells infected with mouse hepatitis virus (MHV-A59). Virology, in the press.

Stern, D.F. and Kennedy, S.I.T. (1980a). The coronavirus multiplication strategy. I. Identification and characterization of virus-specified RNA. Journal of Virology 34, 665-674.

Stern, D.F. and Kennedy, S.I.T. (1980b). Coronavirus Multiplication Strategy. II. Mapping the avian infectious bronchitis virus intracellular RNA species to the genome. Journal of Virology 36, 440-449.

Stohlman, S.A. and Lai, M.M.C. (1979). Phosphoproteins of murine hepatitis viruses. Journal of Virology 32, 672-675.

Sturman, L.S. (1977). Characterization of a coronavirus. I. Structural proteins: effect of preparative conditions on the migration of protein in polyacrylamide gels. Virology 77, 637-649.

Sturman, L.S. and Holmes, K.V. (1977). Characterization of a coronavirus. II. Glycoproteins of the viral envelope: tryptic peptide analysis. Virology 77, 650-660.

Wege, H., Müller, A. and ter Meulen, V. (1978). Genomic RNA of the murine coronavirus JHM. Journal of General Virology 41, 217-227.

Wege, H., Wege, H., Nagashima, K., and ter Meulen, V. (1979). Structural polypeptides of the murine coronavirus JHM. Journal of General Virology 42, 37-47.

Wege, H., Siddell, S.G., Sturm, M. and ter Meulen, V. (1981). Coronavirus JHM. Characterization of intracellular viral RNA, Journal of General Virology, in the press.