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IN VITRO TRANSLATION OF FRACTIONATED VIRUS-SPECIFIC RNA ISOLATED FROM PLASMA OF CHICKEN INFECTED BY AVIAN MYELOBLASTOSIS VIRUS. UNPROCESSED AND PROCESSED MYELOBLASTOSIS-ASSOCIATED VIRUS *ENV*-POLYPEPTIDE PRECURSORS

A. MALÝ

Institute of Molecular Genetics, Czech Academy of Sciences, Flemingovo nám. 2, 166 37 Prague, Czech Republic

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Summary. – The 60 S viral RNA complex isolated from leukaemic plasma of chicken infected by avian myeloblastosis virus (AMV) was denatured, the poly(A)-RNA selected and centrifuged in a linear sucrose density gradient. RNA from each fraction was translated *in vitro* and the products were analyzed by slab polyacrylamide gel electrophoresis (PAGE). Unprocessed primary translation product ($p64^{env}$) of MAV *env* gene from 21 S RNA fraction was immunoprecipitated by anti-gp85 serum. If, however, this RNA was translated in the presence of dog pancreas microsomal membranes (DPM), the processed 92 K MAV glycoprotein precursor ($p92^{env}$) was immunoprecipitated by anti-gp85 serum. This precursor, unlike $p64^{env}$ was resistant to exogenous protease.

Key words: retrovirus; in vitro translation; env precursors

Introduction

The glycoproteins of avian retroviruses – gp85 and gp37 – (Duesberg *et al.*, 1970) are virus-specific and coded by the *env* gene of RNA genome. Both glycoproteins are synthesized from the subgenomic mRNA, namely 21 S RNA in the case of MAV as a single 92 K glycosylated precursor ($gPr92^{env}$) on the rough endoplasmic reticulum of the infected cells (England *et al.*, 1977; Moelling and Hayami, 1977; Purchio *et al.*, 1980; Chen *et al.*, 1981; Gonda *et al.*, 1981). The subgenomic 21 S mRNA of Rous-associated virus 2 (RAV-2), but not the genome fragments was translated upon microinjection into homologous cells transformed by *env*-deficient Bryan strain of Rous sarcoma virus (RSV) (Stacey, 1979). The translation of that mRNA in a heterologous cell-free system, followed by immunoprecipitation of the products with anti-gp85 serum revealed a 64 K polypeptide as the primary unglycosylated RAV *env* gene product

(Pawson *et al.*, 1980; Enrietto *et al.*, 1982). A polypeptide of the same size was also translated *in vitro* from fractionated *env*-containing RSV virion RNA (Pawson *et al.*, 1980).

The population of standard strain of AMV (BAI A), isolated from plasma of infected chickens contains both a replication-defective AMV and replication-competent helper viruses MAV-1 (subgroup A) and MAV-2 (subgroup B) (Ishizaki *et al.*, 1975; Moscovici, 1975). Only a fraction of the envelope glycoproteins of MAVs apparently differs from each other. The oligonucleotide maps of genomic RNA of MAV-1 and MAV-2 are very similar with exception of three oligonucleotides from the *env* region (Duesberg *et al.*, 1980; Schmidt *et al.*, 1982). The cellular locus *myb* (Roussel *et al.*, 1979; Duesberg *et al.*, 1980) was transduced into MAV-1 (Gonda *et al.*, 1985) and substituted there a substantial part of *env* gene. Hence replication-defective AMV arose.

In this work we studied cell-free translation of fractionated viral RNA isolated from chicken leukaemic plasma in a mRNA-dependent rabbit reticulocyte lysate. The *env* gene of MAVs was translated from 21 S viral RNA into a 64 K unglycosylated polypeptide. The co-translational glycosylation of the primary product by DPM to a 92 K glycoprotein was then successfully achieved. Both translated *env*-related polypeptides shared antigenic determinants with gp85. Only the glycosylated product was protected against exogenous protease by DPM unless they were destroyed by the detergent.

Materials and Methods

Antisera. Anti-AMV serum was prepared by intracutaneous injection of rabbits by SDS-lysate of virus isolated from chicken leukaemic plasma. Anti-p27 serum was prepared as described (Malý, 1984). Anti-gp85 serum was a kind gift from Dr. M. Hayman, London (Hayman, 1978). DPM were either a gift from Dr. M. Owen or they were prepared as described (Katz *et al.*, 1977). Trypsin was purchased from Worthington and soy bean trypsin inhibitor from Sigma.

Translation in the presence of DPM. DPM represented 1/15 of the cell-free translation mixture volume and they were present in the reaction mixture from the onset of incubation with ^{35}S-methionine. After 70 mins incubation at 30 °C pancreatic ribonuclease A (100 μg/ml) was added to inhibit further protein synthesis and then each incubation mixture was divided into 3 aliquots. To one aliquot trypsin (0.5 mg/ml) was added, to another one trypsin plus 0.5 % Na-deoxycholate (DOC), and to the last one Hepes (10 mmol/l pH 7.5) were added. After 90 mins incubation at 4 °C soy bean trypsin inhibitor (0.5 mg/ml) was added to all samples.

Isolation of 60 S RNA from chicken leukaemic plasma, denaturation and selection of poly(A)-RNA were described earlier (Malý, 1984). The same applies for the preparation and use of mRNA-dependent rabbit reticulocyte lysate system, immunoprecipitation procedure, and analysis of labelled polypeptides by SDS-PAGE.

Results

The 60 S RNA complex was singled out by fractionation of total viral RNA isolated from chicken leukaemic plasma by sucrose density gradient centrifuga-

tion. This RNA complex was heat-denatured, the poly(A)-RNA isolated and sized by second sucrose density gradient centrifugation. Then each RNA fraction was translated in the mRNA-dependent rabbit reticulocyte lysate system. To detect a 60–65 K polypeptide as unglycosylated *env* gene product ($p64^{env}$) of MAV, aliquots of all reaction mixtures were immunoprecipitated by anti-AMV serum, and the precipitated proteins were analyzed by SDS-PAGE (Fig. 1).

There were 76 K and 180 K polypeptides translated from genome-length RNA in fractions No. 4 and 5. The 76 K polypeptide was a precursor of gag proteins ($Pr76^{gag}$), and the 180 K polypeptide was a precursor containing *gag* and *pol* proteins ($Pr180^{gag\text{-}pol}$). The 76 K polypeptide was also translated from

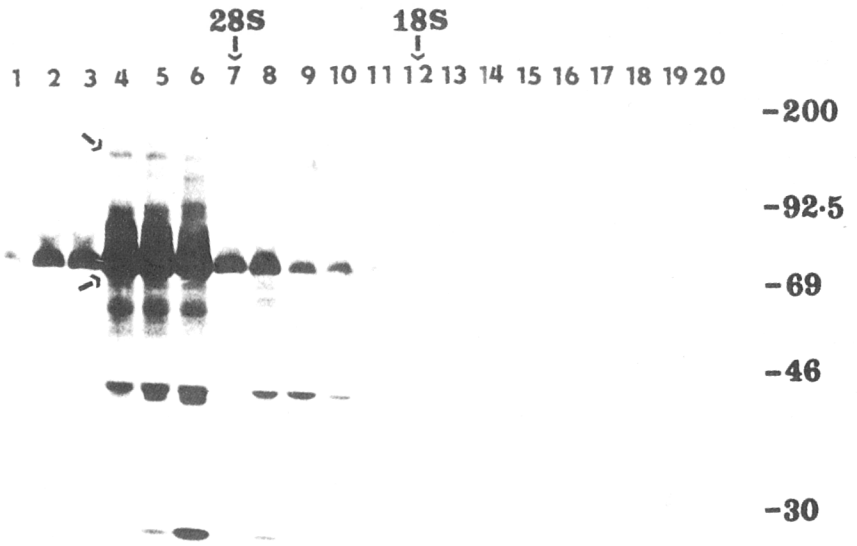

Fig. 1
Proteins translated *in vitro* from fractionated viral RNA immunoprecipitated by anti-AMV serum 7 μg of poly(A)-containing denatured viral 60 S RNA was centrifuged on linear sucrose density gradient (10–25 %) in 50 mmol/l NaCl, 10 mmol/l Tris pH 7.5, 1 mmol/l EDTA, and 0.1 % SDS in SW50.1 rotor (Beckman) at 45 000 rpm for 3 hrs at 20 °C. RNA from each fraction was ethanol-precipitated after adding 3 μg tRNA, washed and translated *in vitro*. 2 μl of products were immunoprecipitated by 1.5 μl of anti-AMV serum and the precipitated proteins were analyzed by SDS-PAGE (7–20 %). Molecular weight values of markers are indicated on the right. Numbers of lanes correspond to numbers of gradient fractions from which the translated RNA originated. Positions of 28 S and 18 S rRNA markers are indicated. Arrows point to $Pr76^{gag}$ and $Pr180^{gag\text{-}pol}$.

22–26 S RNA (fractions No. 8–10) and immunoprecipitated, suggesting that the anti-AMV serum contained also antibodies directed against reverse transcriptase protein, and hence they immunoprecipitated p76^{pol} (Malý, 1984). Pr180$^{gag\text{-}pol}$ was not detected in fractions No. 8–10.

A 60–65 K polypeptide was not easily seen on the fluorogram of products of *in vitro* translation of 21 S viral RNA (fractions No. 10–11) without immunoprecipitation (data not shown) and after immunoprecipitation with anti-AMV serum (Fig. 1). In order to detect it, the anti-gp85 serum was used (Fig. 2). A 64 K

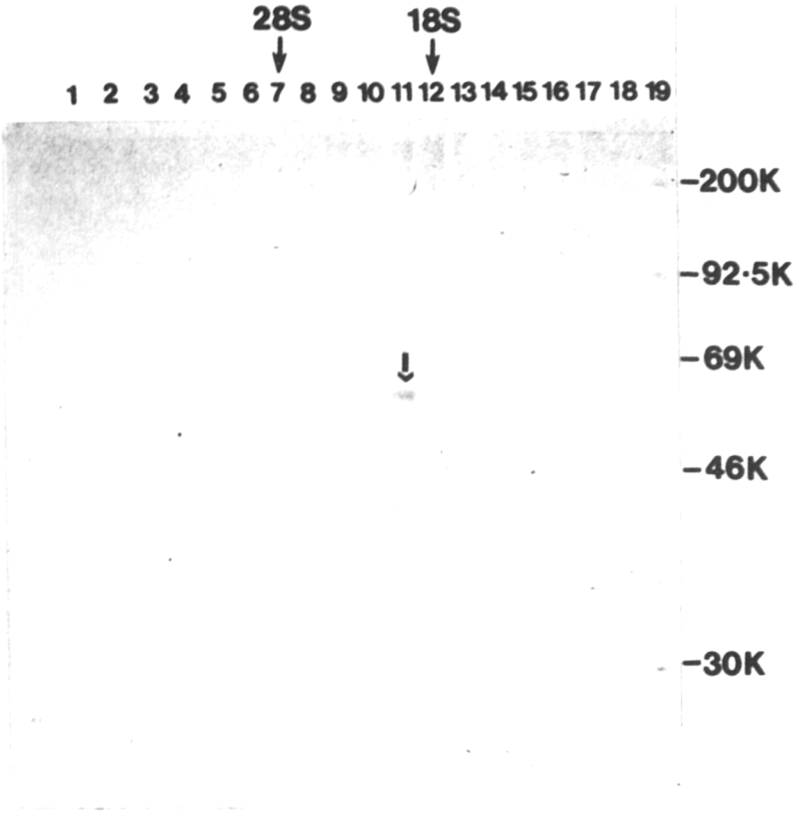

Fig. 2

Proteins with glycoprotein antigenic determinants translated *in vitro* from fractionated viral RNA 2 μl aliquots of the *in vitro* translation products (see legend to Fig. 1) were clarified by immunoprecipitation by anti-p27 serum and the supernatants were immunoprecipitated by anti-gp85 serum (10 %). Products were analyzed by SDS-PAGE (10 %). Arrow points to the MAV glycoprotein precursor polypeptide. For the rest of the legend see Fig. 1.

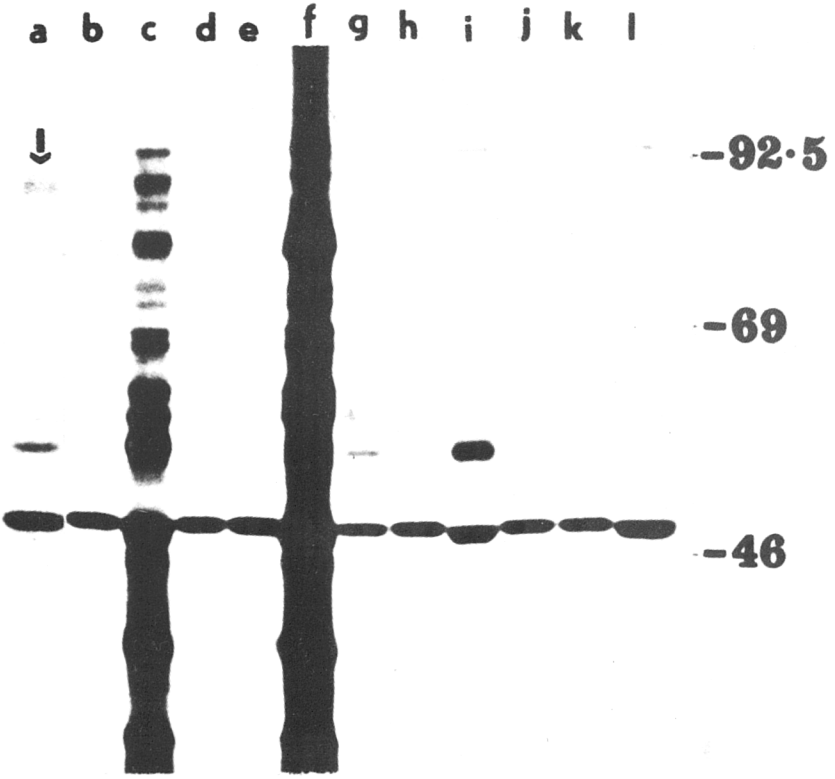

Fig. 3

In vitro translation of unglycosylated and glycosylated MAV glycoprotein products

7 μg of poly(A)-containing viral RNA was centrifuged and fractionated as described in Fig. 1. Fractions No. 10 and 11 containing 21 S RNA were pooled, ethanol-precipitated, washed, and translated *in vitro* with and without DPM. Products (5 μl) were analyzed by SDS-PAGE (10 %). Translation of 21 S RNA with (lanes a–c) and without DPM (lanes d–f). Endogenous reaction with (lanes g–i) and without DPM (lanes j–l). Products were treated with trypsin (lanes a, d, g, j), and with trypsin plus DOC (lanes b, e, h, k). Controls (lanes c, f, i, l). Arrow points to the position of glycosylated precursor of MAV glycoprotein. Lane a was exposed for a longer time. For the rest of the legend see Fig. 1.

polypeptide was clearly detected in fractions No. 10 and 11. Because it was translated from 21 S viral RNA and shared antigenic determinants with viral gp85, it represented probably p64^{env} of MAV.

In order to process the primary translational *env* product of MAV *in vitro*, DPM were employed. The RNA fractions No. 10 and 11 of parallel sucrose gradient (as depicted in Fig. 1) were pooled, ethanol-precipitated and translated *in vitro* with (Fig. 3, lanes a–c) and without (lanes d–f) DPM. As a control of contribution of the system itself and DPM, respectively, served incubations without RNA in the presence (lanes g–i) and in the absence (lanes j–l) of DPM. To detect polypeptides protected by DPM, parts of all reaction mixtures were digested with trypsin and with trypsin plus DOC, respectively. There was seen a 93 K polypeptide translated in the system in the absence of DPM (lane 1), which was sensitive to protease (lane j). The system with DPM synthesized moreover a 55 K polypeptide (lane i), resistant to trypsin (lanes a, g), but sensitive to trypsin plus DOC (lanes b, h). The nature of these endogenous bands was not investigated further.

The translation of 21 S viral RNA *in vitro* in the absence of DPM yielded many polypeptides (lane f). We assume that the *env* gene product might be obscured by one of the *env*-unrelated products. All of them were sensitive to protease treatment (lane d). The efficiency of translation of this RNA was lowered by the presence of DPM (lanes f, c), but among others a 92 K polypeptide was

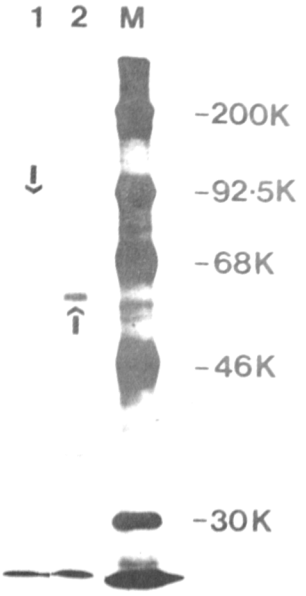

Fig. 4
Precursors of MAV glycoprotein translated *in vitro* and immunoprecipitated with anti-gp serum
15 μl aliquots of the *in vitro* translation products (Fig. 3, lanes c, f) were immunoprecipitated by 1.5 μl of anti-gp serum and analyzed by SDS-PAGE (10 %) (lanes 1, 2). Molecular weight markers (lane M). The downward pointing arrow indicates p92^{env}, the upward pointing arrow indicates p64^{env}. For the rest of the legend see Fig. 1.

translated in that case (lane c). It was resistant to trypsin treatment (lane a), but only in the absence of detergent (lane b).

In order to show that this polypeptide is *env*-related and represents the primary translational *env* product of MAV from 21 S viral RNA, processed by *in vitro* glycosylation, parts of the *in vitro* translation mixtures were immunoprecipitated by anti-gp85 serum (Fig. 4). Both the 92 K glycoprotein translated in the presence of DPM (lane 1), and the 64 K polypeptide translated in the absence of DPM (lane 2) contained antigenic determinants of retroviral glycoprotein proving that MAV $p64^{env}$ was translated *in vitro* from spliced viral 21 S *env* mRNA, and that the primary translational *env* product was glycosylated *in vitro* to the $p92^{env}$ by processing machinery of DPM.

Discussion

For the study of *env* protein products of MAV we have employed an experimental strategy similar to that used for the study of $p76^{pol}$ (Malý, 1984). Viral RNA was isolated from chicken plasma, fractionated and *in vitro* translated. When anti-gp serum was used for immunoprecipitation of the translated proteins from individual RNA fractions a 64 K polypeptide was detected in fractions with 21 S RNA.

According to the signal hypothesis (Blobel and Dobberstein, 1975) all proteins which have to be transferred across a membrane (e. g. cellular secretory proteins, viral glycoproteins etc.) contain a sequence of 20 mainly hydrophobic amino acids at the N-terminus of the nascent chain which is called the signal peptide (Milstein *et al.*, 1972). The mRNAs for those proteins contain a unique sequence of codons. The signal peptide of RSV glycoprotein precursor is probably encoded partially in *gag* and mainly in *env*, the latter overlapping with *pol* gene (Dickson *et al.*, 1982). The translational products of these mRNAs should be vectorially discharged, unfolded (Eilers and Schatz, 1988) and segregated in the intravesicular space, where they should be processed through enzymatic modifications confined to the intracisternal space, e. g. endoproteolytic removal of the signal sequence from the nascent polypeptide chain and glycosylation. When avian retrovirus-infected cells have been labelled in the presence of glycosylation inhibitors tunicamycin and 2-deoxyglucose (Diggelman, 1979; Stohrer and Hunter, 1979), only a 57 K *env*-related polypeptide was detected. The putative *env* signal sequence was probably cleaved from the nascent polypeptide and the glycosylation was prevented by the inhibitors.

However, the $p64^{env}$ translated here in the mRNA-dependent reticulocyte lysate unable of post-translational modification of synthesized proteins (Ploegh *et al.*, 1979), contained probably the whole signal sequence. Viral RNA suitable for its translation should be a properly spliced subgenomic *env* mRNA of MAV(s). As a molecular proof of $p64^{env}$ processibility the DPM were included into the *in vitro* translation mixture as it was elaborated for the experiments with

vesicular stomatitis virus (Rothman and Lodish, 1977) and used with RAV-2 viral RNA (Enrietto *et al.*, 1982). DPM caused a decrease of mobility of the precursor in the gel and the 92 K protease-resistant glycoprotein with antigenic determinants of gp85 was detected unless DPM were destroyed by the detergent. Because resistance to proteolysis is at present the only rigorous proof of vectorially discharged polypeptide chains (Sabatini and Blobel, 1970), and moreover gPr92^{env} in comparison with gp85 was shown to be more sensitive to trypsin treatment (Klamenz and Diggelman, 1978), the protein translated *in vitro* in the presence of DPM was the glycosylated p92^{env} of MAV.

References

Blobel, G., and Dobberstein, B. (1975): Transfer of proteins across membranes I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane bound ribosomes of murine myeloma. *J. Cell Biol.* **67**, 835–851.

Chen, J. H., Hayward, W. S., and Moscovici, C. (1981): Size and genetic content of virus-specific RNA in myeloblasts transformed by avian myeloblastosis virus (AMV). *Virology* **110**, 128–136.

Dickson, C., Eisenman, R., Fan, H., Hunter, E., and Teich, N. (1982): Protein biosynthesis and assembly, pp. 513–648. In R. Weiss, N. Teich, H. Varmus and J. Coffin (Eds): *RNA tumor viruses,* Cold Spring Harbor Laboratory, Cold Spring Harbor.

Diggelman, H. (1979): Biosynthesis of an unglycosylated envelope glycoprotein of Rous sarcoma virus in the presence of tunicamycin. *J. Virol.* **30**, 799–804.

Duesberg, P. H., Martin, G. S., and Vogt, P. K. (1970): Glycoprotein components of avian and murine RNA tumor viruses. *Virology* **41**, 631–646.

Duesberg, P. H., Bister, K., and Moscovici, C. (1980): Genetic structure of avian myeloblastosis virus, released from transformed myeloblasts as a defective virus particle. *Proc. natn. Acad. Sci. U. S. A.* 77, 5120–5124.

Eilers, M., and Schatz, G. (1988): Protein unfolding and the energetics of protein translocation across biological membranes. *Cell* **52**, 481–483.

England, J. M., Bolognesi, D. P., Dietzschold, B., and Halpern, M. S. (1977): Evidence that a precursor glycoprotein is cleaved to yield the major glycoprotein of avian tumor virus. *J. Virol.* **21**, 810–814.

Enrietto, P. J., Purchio, A. F., and Erikson, R. L. (1982): Synthesis of the viral glycoprotein of Rous-associated virus-2. *J. Virol.* **43**, 756–760.

Gonda, T. J., Sheiness, D. K., Fanshier, L., Bishop, J. M., Moscovici, C., and Moscovici, M. G. (1981): The genome and intracellular RNAs of avian myeloblastosis virus. *Cell* **23**, 279–290.

Hayman, M. J. (1978): Viral polyproteins in chick embryo fibroblasts infected with avian sarcoma leukosis viruses. *Virology* **85**, 241–252.

Ishizaki, R., Langlois, A. J., and Bolognesi, D. P. (1975): Isolation of two subgroup-specific leukemogenic viruses from standard avian myeloblastosis viruses *J. Virol.* **15**, 906–912.

Katz, F. N., Rothman, J. E., Lingappa, V. R., Blobel, G., and Lodish, H. F. (1977): Membrane assembly *in vitro*: synthesis, glycosylation, and assymetric insertion of a transmembrane protein. *Proc. natn. Acad. Sci. U. S. A.* **74**, 3278–3282.

Klemenz, R., and Diggelmann, H. (1978): The generation of the two envelope glycoproteins of Rous sarcoma virus from a common precursor polypeptide. *Virology* **85**, 63–74.

Malý, A. (1984): *In vitro* translation of fractionated RNA isolated from chicken leukaemic plasma virus (AMV). I. A 76 K pol-related polypeptide. *Folia biol. (Prague)* **30**, 404–411.

Milstein, C., Brownlee, C. G., Harrison, T. M., and Mathews, M. B. (1972): A possible precursor of immunoglobulin light chains. *Nature New Biol.* **239**, 117–120.

Moelling, K., and Hayami, M. (1977): Analysis of precursors to the envelope glycoproteins of avian RNA tumor viruses in chicken and quail cells. *J. Virol.* **22**, 598–607.

Moscovici, C. (1975): Leukaemic transformation with avian myeloblastosis virus: present status. *Curr. Top. Microbiol. Immunol.* **71**, 79–95.

Pawson, T., Mellon, P., Duesberg, P. H., and Martin, G. S. (1980): *Env* gene of Rous sarcoma virus: identification of the gene product by cell-free translation. *J. Virol.* **33**, 993–1003.

Ploegh, H. L., Cannon, L. E., and Strominger, J. L. (1979): Cell-free translation of the mRNAs for the heavy and light chains of HLA-A and HLA-B antigens. *Proc. natn. Acad. Sci.* **76**, 2273–2277.

Purchio, A. F., Jovanovich, S., and Erikson, R. L. (1980): Sites of synthesis of viral proteins in avian sarcoma virus-infected chicken cells. *J. Virol.* **35**, 629–636.

Rothman, J. E., and Lodish, H. F. (1977): Synchronised transmembrane insertion and glycosylation of a nascent membrane protein. *Nature* **269**, 775–780.

Sabatini, D. D., and Blobel, G. (1970): Controlled proteolysis of nascent polypeptides in rat liver cell fractions. II. Location of the polypeptides in rough microsomes. *J. Cell Biol.* **45**, 146–157.

Schmidt, E. V., Keene, J. D., Linial, M., and Smith, R. E. (1982): Association of 3' terminal RNA sequences with avian leukosis viruses causing a high incidence of osteopetrosis. *Virology* **116**, 163–180.

Stacev, D. W. (1979): Messenger activity of virion RNA for avian leukosis viral envelope glycoprotein. *J. Virol.* **29**, 949–956.

Stohrer, R., and Hunter, E. (1979): Inhibition of Rous sarcoma virus replication by 2-deoxyglucose and tunicamycin: Identification of unglycosylated *env* gene product. *J. Virol.* **32**, 412–419.