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Equine Arteritis Virus-induced Polypeptide Synthesis

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SUMMARY

Intracellular virus-specific proteins induced by equine arteritis virus (EAV) have been compared with *in vitro* translation products of virion and intracellular EAV RNAs. In infected BHK-21 cells, the two major virion proteins (C and E1) and polypeptides with mol. wt. of 60000 (p60), 42000 (p42) and 30000 (p30) were found. There were no indications that the viral proteins were processed from a larger precursor as shown by pulse-chase, amino acid analogue and protease inhibitor experiments. The six polyadenylated RNAs that occur in EAV-infected cells were isolated and translated in an mRNA-dependent reticulocyte cell-free system. Translation of RNA6 resulted in the appearance of a product having the mol. wt. (14000) of the nucleocapsid protein (C). EAV genomic RNA was translated into proteins of mol. wt. 30000 and 200000, while RNA1, the intracellular homologue of genomic RNA only encoded p30. The absence of large precursor molecules in infected cells and the results from the *in vitro* translation experiments both suggest that at least some of the proteins are primary translation products.

INTRODUCTION

Equine arteritis virus (EAV) is a small enveloped positive-stranded RNA virus. It has a diameter of 60 ± 13 nm and consists of an isometric core of about 35 nm which is surrounded by an envelope carrying ring-like structures (Horzinek, 1981). On the basis of morphology, size of the virion and properties of the RNA genome, EAV has been classified as a non-arthropod-borne member of the family Togaviridae (Porterfield *et al.*, 1978), the only member of the recently established genus arterivirus (Westaway *et al.*, 1985). In horses, EAV may cause necrosis of the muscle cells surrounding small arteries (Jones *et al.*, 1957) and abortion in pregnant mares (Doll *et al.*, 1957). Serological evidence suggests that most infections do not result in clinical signs and pass unnoticed.

The virion contains three structural polypeptides: a nucleocapsid protein (C) of 12000 mol. wt. (12K), a 14K non-glycosylated envelope protein (E1) and a minor 21K glycosylated envelope protein (E2) (Zeegers *et al.*, 1976). In addition, a number of glycoproteins, migrating as a complex in the mol. wt. range between 28K and 40K were found in purified virus preparations but, however, may be of cellular origin. Since the sum of the mol. wt. of the confirmed and putative virion polypeptides amounts to between 75K and 87K, there is sufficient capacity available in the EAV genome (mol. wt. 4.3×10^6 ; van Berlo *et al.*, 1982; van der Zeijst *et al.*, 1975) to code for a considerably greater complement of proteins than has been identified so far. In addition to genome-sized RNA, five polyadenylated virus-specific RNA species were found in EAV-infected cells, having mol. wt. of 1.3, 0.9, 0.7, 0.3 and 0.2, all $\times 10^6$. The coding potential of these RNAs, however, is not known.

We have translated the EAV-specific polyadenylated subgenomic RNAs *in vitro* and compared their products to those found in EAV-infected cells. Both the results from the *in vitro* translation experiments and the absence of large protein precursor molecules in infected cells suggest that some of the EAV proteins are primary translation products.

METHODS

Cells and viruses. Stocks of the Bucyrus strain of EAV (Doll *et al.*, 1957) were prepared as described previously (van Berlo *et al.*, 1980, 1982). The virus was grown in BHK-21 cells for purification or biosynthesis experiments and was plaque-titrated on Vero cells (van Berlo *et al.*, 1980, 1982). Sindbis virus was obtained from Dr S. Schlesinger (Washington University School of Medicine, St. Louis, Mo., U.S.A.).

Radioactive labelling of intracellular proteins. BHK-21 cells grown in 35 mm² tissue culture dishes were infected with EAV at 40 °C as described previously (van Berlo *et al.*, 1980, 1982). At various times after infection, the cells were washed twice with phosphate-buffered saline (PBS) and pulse-labelled for 1 h in 1 ml methionine-deficient MEM supplemented with 5% foetal calf serum (MEM-5% FCS) containing 25 µCi [³⁵S]methionine (800 Ci/mmol). For pulse-chase experiments, the medium was removed after a 0.5 h labelling period and the cells were rinsed with PBS and further incubated in DMEM-10% FCS containing 1 mg/ml unlabelled methionine. To label glycoproteins, cells were incubated in MEM-5% FCS containing one-tenth the standard amount of glucose and 50 µCi D-[2-³H]mannose or D-[6-³H]glucosamine hydrochloride (16 and 38 Ci/mmol, respectively; all radiochemicals were from Amersham). Cells were lysed in 150 µl TES buffer (0.02 M-Tris-HCl, 1 mM-EDTA, 0.1 M-NaCl, pH 7.4), containing 0.5% Triton X-100, 0.5% 1,5-naphthalene disulphonate disodium salt and 2 mM-phenylmethylsulphonyl fluoride (lysis buffer). The cell lysate was clarified by centrifugation for 2 min at 10000 g and the supernatant was kept at -70 °C. The membrane pellet including the nuclei was washed twice with TES buffer supplemented with 0.1% Triton X-100 and resuspended in SDS-PAGE sample buffer. Proteins were analysed by SDS-PAGE as described below. Tunicamycin was a gift from R. L. Hamill, Eli Lilly Research Laboratories, Indianapolis, Ind., U.S.A.

Isolation and purification of virus-specific intracellular RNAs. Total RNA from EAV-infected BHK-21 cells grown in 20 roller bottles (1170 cm² surface) was isolated essentially as described previously (Rottier *et al.*, 1981). Briefly, the bottles were cooled in ice water at 8 h post-infection, the cells were washed with ice-cold PBS and lysed with a total volume of 170 ml lysis buffer. The lysate was clarified by centrifugation for 25 min at 3000 g and 5 °C and mixed with 170 ml 2% SDS-7 M-urea in TES buffer. RNA was then isolated by repeated phenol extractions and precipitated with ethanol. The yield was about 90 mg of RNA from which 1.3 mg polyadenylated [poly (A)⁺] RNA was obtained after oligo(dT)-cellulose column chromatography, using RNA from infected cells labelled with ³²P, in the presence of actinomycin D as a marker. The poly(A)⁺ RNA-containing fraction was dissolved in 10 mM-sodium phosphate pH 7.0 containing 1 mM-EDTA, 0.25% SDS and 10 µg bromophenol blue per ml, heated for 5 min at 56 °C, rapidly cooled on ice and run in a horizontal 1% agarose-6 M-urea slab gel. The individual RNAs were separated as described previously (Rottier *et al.*, 1981).

In vitro translation of RNAs. The individual RNAs were added to an mRNA-dependent reticulocyte cell-free system prepared as described by Pelham & Jackson (1976), using a final RNA concentration of 0.1 mg/ml. Translation was allowed to proceed for 80 min at 30 °C. The reaction mixture was then cooled on ice and samples were analysed by SDS-PAGE, either directly or after immunoprecipitation with an antiserum.

Immunoprecipitation of proteins. Samples of 10 µl from the reticulocyte translation system were diluted with 60 µl lysis buffer and were incubated for 15 h at 4 °C with 10 µl hamster anti-EAV serum, prepared as described previously (van Berlo *et al.*, 1980). Non-specific precipitation was reduced by the addition of 3 M-KCl to a final concentration of 0.5 M immediately before the addition of 20 µl rabbit anti-hamster IgG serum (Miles-Yeda, Rehovot, Israel). These mixtures were then incubated at 4 °C for 24 h. Immune precipitates were collected by centrifugation, washed twice with TES buffer supplemented with 0.1% Triton X-100 and analysed by gel electrophoresis. Samples of 5 µl were spotted on Whatman 3MM filter paper discs, which were washed twice in 5% TCA, once in ether-ethanol, and once in ether before they were dried and radioactivity was counted.

RESULTS

Protein synthesis in EAV-infected cells

EAV-infected cells were examined at 2 h intervals during a 14 h period following infection at a multiplicity of 30 p.f.u./cell. Infected and mock-infected cells were labelled with [³⁵S]methionine for 1 h. Before addition of label, the culture medium was collected and assayed for infectious virus. Maximum titres of infectious virus (about 10 p.f.u./cell) were reached at 12 h post-infection (Fig. 1). At the end of the labelling period the cells were harvested, lysed, and the cytoplasmic extracts were analysed. The amount of immunoprecipitated virus proteins decreased sharply during the exponential phase of virus growth (Fig. 1).

Identification of intracellular viral proteins

Cytoplasmic extracts and membrane pellets labelled between 7 and 8 h after infection were analysed by SDS-PAGE (Fig. 2). In the cytoplasmic fraction from infected cells proteins with

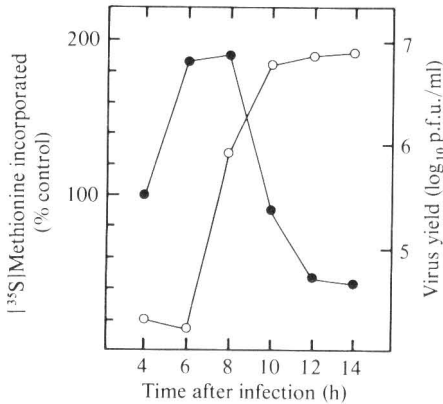


Fig. 1

Fig. 1. Release of infectious EAV into culture medium (○) and rate of virus protein synthesis (●) in EAV-infected BHK-21 cells. BHK-21 cells were infected with EAV at a multiplicity of 30 p.f.u./cell and labelled with [³⁵S]methionine. Medium samples were plaque-titrated to determine the yield of extracellular infectious virus. The amount of incorporated radioactivity of virus-specific proteins, immunoprecipitated from the cell lysates with hamster anti-EAV serum, was measured after TCA precipitation.

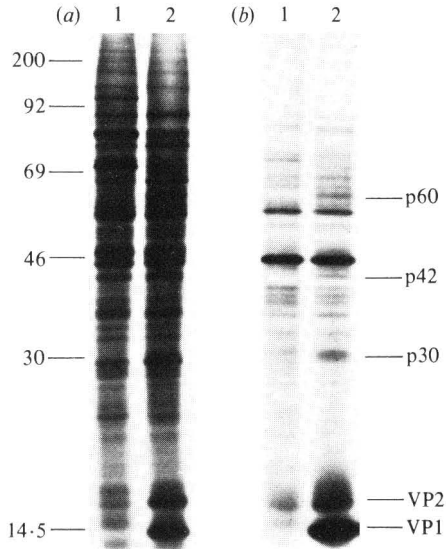


Fig. 2

Fig. 2. Analysis of polypeptides in mock-infected (lanes 1) and EAV-infected (lanes 2) BHK-21 cells. Infection and labelling were performed as described in Fig. 1. Cytoplasmic lysates (a) and membrane pellets (b) from equal numbers of cells, labelled from 7 to 8 h post-infection with [³⁵S]methionine for 1 h were electrophoresed in parallel. Mol. wt. standards ($\times 10^{-3}$) and EAV polypeptides are indicated.

mol. wt. of 14K and 16K were detected which were absent from mock-infected cells. The membrane pellets contained three additional unique proteins of 30K, 42K and 60K. The 14K and 16K proteins co-migrated respectively with the capsid proteins (C) and the envelope protein (E1) of purified virus (not shown). No equivalent to E2 (21K) and the glycoproteins in the mol. wt. range between 28K and 40K were found.

The possibility that the EAV structural proteins might arise by post-translational processing from larger precursor proteins was investigated. Infected BHK-21 monolayers were pulse-labelled for 0.5 h with [³⁵S]methionine at 7 h post-infection and excess unlabelled methionine was added for various times up to 2 h. No effect on the relative amounts of the labelled virus-specific proteins was observed during the chase (results not shown). Even after a 5 min pulse the polypeptide profile was identical to that obtained after a 1 h labelling period. To investigate the possibility that processing was extremely quick, infected cells were treated with the amino acid analogue canavanine, the protease inhibitors TPCK, TLCK or ZnCl₂. These treatments were effective in causing the accumulation of large precursor molecules in Sindbis virus-infected cells but not in EAV-infected cells (results not shown). Attempts to label the virus-induced proteins in infected cells with [³H]mannose or [³H]glucosamine were unsuccessful.

In vitro translation of intracellular EAV-specific RNAs

Translation experiments were carried out in a reticulocyte system with polyadenylated RNAs from infected and uninfected cells. No virus-related products could be identified among the numerous polypeptides upon direct analysis (Fig. 3b). Immunoprecipitation was performed to enhance the specific signals above the background. The immune serum had been obtained from

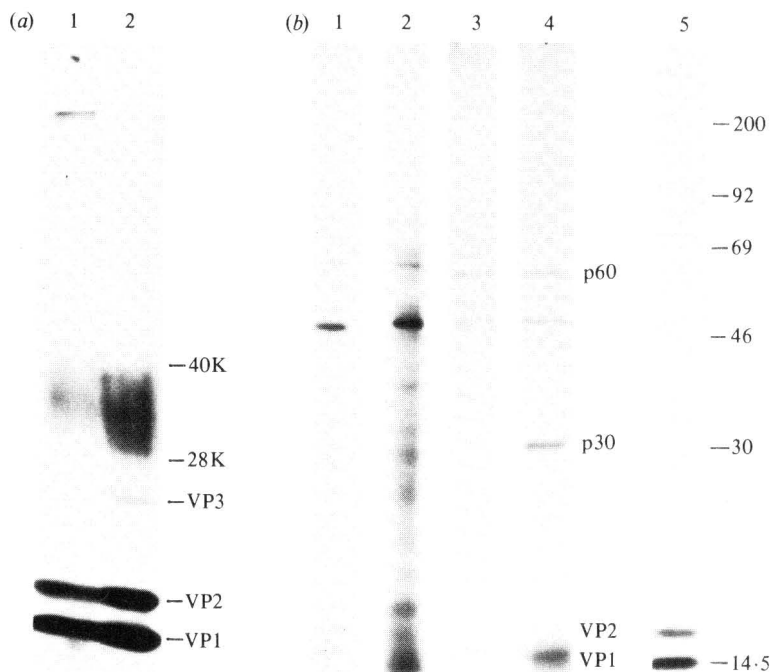


Fig. 3. Analysis of the *in vitro* translation products of poly(A)⁺ selected RNA isolated from EAV-infected and mock-infected cells. (a) Specificity of a hamster antiserum directed against EAV proteins (lane 2) using [³⁵S]methionine-labelled virus as antigen in immunoprecipitation (lane 1). The antiserum recognizes VP1, VP2 and proteins in the mol. wt. range between 28K and 40K. (b) Translation products of total poly(A)⁺ selected RNA, isolated from mock-infected cells (lanes 1 and 3) and EAV-infected cells (lanes 2 and 4). Samples of the lysates were used either directly (lanes 1 and 2) or after immunoprecipitation with the anti-EAV hamster serum (lanes 3 to 5). A rabbit anti-hamster antiserum was employed for precipitation of the immune complexes. Lane 5, virion proteins. Standard and EAV proteins are indicated.

hamsters immunized with lysates of EAV-infected BHK-21 cells; its specificity is shown in Fig. 3(a). The virion proteins C and E1 and a number of glycoproteins in the mol. range 28K to 40K were immune-precipitated but VP3 was not recognized. Translation of poly(A)⁺ RNA isolated from infected cells gave rise to polypeptides with mol. wt. of 14K, 30K and 60K that were recognized by the antiserum. The 14K and 30K polypeptides were absent from the translation products of RNA isolated from mock-infected cells. They co-migrated with the EAV nucleocapsid protein and the intracellular 30K protein found in membrane-containing fractions from infected cells (see Fig. 2), respectively. The 60K polypeptide, however, co-migrated with a polypeptide encoded by polyadenylated RNA from mock-infected cells (Fig. 3b). E1, E2 and p42 (see Fig. 2) were not detected in these translation experiments.

In vitro translation of individual EAV-specific RNAs

EAV-specific intracellular poly(A)⁺ RNAs isolated from infected cells were separated, and enriched preparations of RNA1 and RNA6 were obtained (Fig. 4). These isolated RNAs were then translated in a reticulocyte lysate system in the presence of [³⁵S]methionine and the products analysed by SDS-PAGE, either directly or after immune precipitation. Genomic RNA was included for comparison; the results are shown in Fig. 5. No proteins were recognized by the immune serum in lysates incubated without added RNA. RNA6 preparations induced translation of one main polypeptide with a mol. wt. of 14K, which co-migrated with the C protein of purified virus. RNA1, the intracellular homologue of genomic RNA, produced a 30K

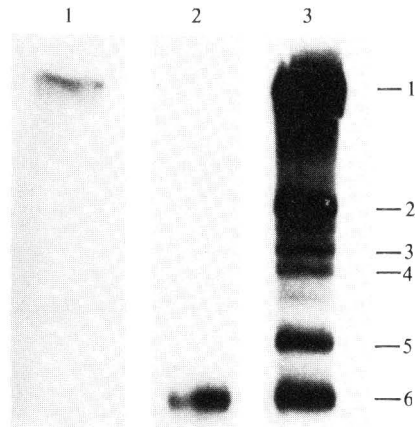


Fig. 4. Analysis of purified EAV-specific intracellular RNAs. Virus-specific intracellular RNA was prepared at 8 h post-infection from BHK-21 cells. A sample of RNA extracted from EAV-infected cells labelled with $^{32}\text{P}_i$ was added as a marker. The poly(A)-containing fraction was isolated using oligo(dT)-cellulose chromatography and electrophoresed in an agarose/urea gel. Bands corresponding to RNA1 and RNA6 were excised from the gel and RNA was eluted from the gel slices. Samples of the purified fractions were re-electrophoresed (lanes 1 and 2 respectively) after glyoxal denaturation to assess their purity, with unfractionated poly(A)-containing RNAs serving as a marker (lane 3).

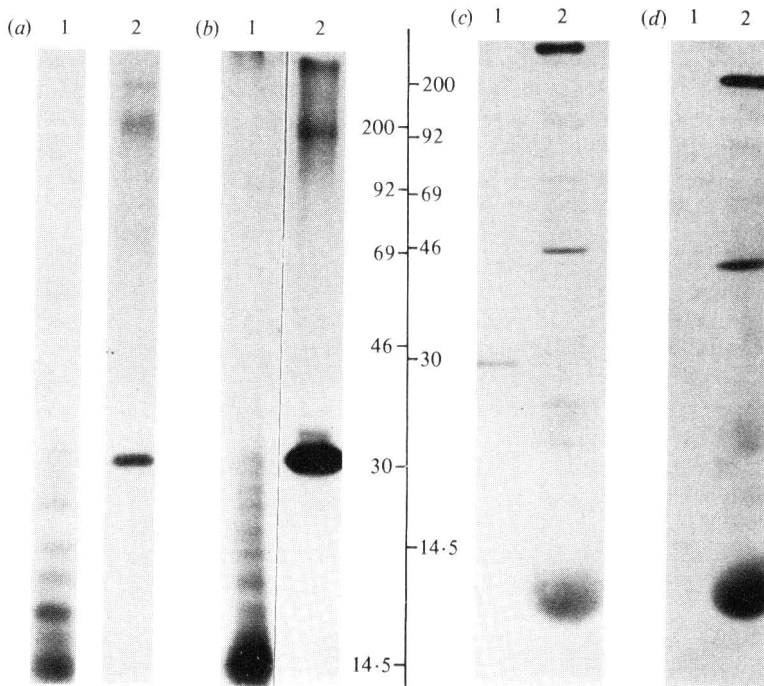


Fig. 5. Analysis of the *in vitro* translation products of the EAV genome, and the intracellular species RNA1 and RNA6. Isolated RNA (1 μg) was added to a reticulocyte system, in the presence of ^{35}S methionine. Samples of the lysate were taken for direct analysis or were used to prepare immunoprecipitates (see legend to Fig. 3). (a) Direct analysis of products of RNA6 (lane 1) and the genome (lane 2). (b) as (a), but immunoprecipitated before electrophoresis. (c) Products of RNA1 immunoprecipitated (lane 1) or not (lane 2) before electrophoresis. (d) as (c), but no RNA was added to the lysate. Mol. wt. standards are indicated.

protein which became apparent only after prolonged exposure of the gel. Translation of the genomic RNA also resulted in the synthesis of a 30K protein in addition to a high mol. wt. (200K) species.

DISCUSSION

In this first report on protein synthesis in EAV-infected cells we describe the identification of five virus-induced polypeptides of mol. wt. 14K, 16K, 30K, 42K and 60K. As judged from their co-migration during SDS-PAGE with virion proteins, the 14K and 16K species probably correspond to C and E1, respectively. We consider these slightly higher molecular weight values more accurate than those described previously (Zeegers *et al.*, 1976). The 30K, 42K and 60K polypeptides occur mainly in association with membranes and the nuclear fraction; these structures have been reported before to be associated with particular proteins during togavirus replication (Westaway, 1980). No precursor-product relationships could be demonstrated; in pulse-chase experiments none of the polypeptides appeared to undergo any modification, even after very short pulses with [³⁵S]methionine. It is consistent with these findings that no high mol. wt. polypeptide accumulated in the presence of inhibitors of proteolysis or canavanine. When infected cells were radioactively labelled with mannose or glucosamine, no label was incorporated in any of the virus-specific proteins. However, preliminary tunicamycin experiments have indicated that this drug strongly inhibits the production of infectious virus (results not shown). Purified virus contains low levels of E2 (Zeegers *et al.*, 1976), a glycoprotein. In infected cells we have not detected this protein which may be essential for virus maturation or infectivity.

Upon infection of cells with EAV the synthesis of five polyadenylated virus-specific RNAs is induced in addition to the genome RNA (van Berlo *et al.*, 1982). Using RNase T1 fingerprinting analysis, we have been able to show that the subgenomic RNAs form a nested set (M. F. van Berlo *et al.*, unpublished results). *In vitro* translation of RNA6, the smallest RNA (which can be obtained uncontaminated by other species) resulted in the synthesis of a virus-specific protein, recognized by an EAV antiserum in immunoprecipitation, which co-migrated with the 14K nucleocapsid protein. As indicated above, RNA5 is an extension of RNA6. The unique region of RNA5 is only 0.3 kb in length, which is insufficient to encode one of the other proteins described above. This suggests to us the existence of overlapping reading frames in RNA5 and RNA6.

Among the three proteins found in the membrane-containing fraction of infected cells, p30 co-migrates with an *in vitro* translation product of RNA1 and of genomic RNA. It remains unclear whether this protein is encoded by genomic RNA or by RNA degraded during the *in vitro* translation. The 30K protein could originate from the same translational unit as the 200K polypeptide by partial readthrough of the p30 stop codon, a strategy of gene expression used by some plant viruses (e.g. carnation mottle virus: Guilley *et al.*, 1985). The virus specificity of the 60K protein is questionable since a protein of identical size was also found among the translation products of polyadenylated RNAs from mock-infected cells. It may, however, be a virus-induced cellular protein which would explain the existence of RNA⁻ protein⁺ *ts* mutants of EAV (van Berlo *et al.*, 1980). The inhibition of the production of infectious EAV (but not of Semliki Forest virus) in cells pre-irradiated with u.v. (M. C. Horzinek & M. Weiss, unpublished results) is compatible with the assumption that some cellular function is required for EAV replication. No virus-specific translation products of RNA5, RNA4, RNA3 and RNA2 were found, probably due to a low intrinsic efficiency of translation.

In order to resolve the information contained in these viral RNAs, cell-free translations will have to be done using purified and concentrated preparations for example obtained by hybrid selection with cDNA clones. These studies, as well as the elucidation of cellular functions during EAV replication, are presently under way.

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