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Translation of Encephalomyocarditis Viral RNA in Oocytes of *Xenopus laevis*

(picornavirus/frog oocytes/protein synthesis/gel electrophoresis/peptide analysis)

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ABSTRACT **RNA from encephalomyocarditis virus was injected into oocytes of *Xenopus laevis*. After incubation of the oocytes in [^{35}S]methionine, polyacrylamide gel electrophoresis showed that a new series of polypeptides had been synthesized. They were identical in size to the polypeptides that appeared in ascites cells after infection with this virus. Electrophoretic and chromatographic analysis of the methionine-containing tryptic peptides from three of the induced polypeptides confirmed that they were virus-specific. All of the bands that appeared in ascites cells after infection also appeared in oocytes after injection of RNA from the virus. We conclude that *Xenopus* oocytes translate a mammalian viral mRNA faithfully and extensively, and perform normal post-translational modifications.**

Cell organelles or solutions of macromolecules can be injected into living oocytes of *Xenopus laevis* (1, 2). When messenger RNA for globin or calf-lens crystallin is injected into such oocytes, it is translated (2, 3). Gurdon *et al.* (2) estimated that each molecule of injected globin mRNA is translated once every 5–10 min for 25 hr. Since the purest sources of natural mRNA currently available are RNA viruses, this paper investigates the ability of oocytes to translate a viral mRNA. Encephalomyocarditis (EMC), a picornavirus, was chosen for this study because the virus-specific proteins synthesized in infected cells have been identified (4), and the activity of the virion RNA as a messenger has been studied *in vitro* (5–9). EMC RNA has only been partially translated *in vitro* (7, 8), and its translation products *in vivo* undergo an extensive series of post-translational modifications.

MATERIALS AND METHODS

Ascites Cells and EMC Virus. Strain K_2 of EMC virus (10) was grown in Krebs II ascites cells (11) and purified either by the procedure of Burness (12) or, more recently, by that of Kerr and Martin (13). RNA was extracted from purified virus by the method of Kerr *et al.* (14), precipitated with ethanol, dialyzed against oocyte injection medium (2), and stored at −70°. One sample of EMC RNA used here was kindly donated by Dr. M. B. Matthews of the MRC Laboratory of Molecular Biology, Cambridge.

Oocytes of Xenopus laevis were injected and cultured by published methods (1, 2).

Abbreviation: EMC, encephalomyocarditis (virus).

Isotopic Labeling of Oocytes, Ascites Cells, and Virus. [^{35}S]-Methionine was purchased from the Radiochemical Centre, Amersham, Bucks, at 10–15 Ci/mmol or was prepared from *Escherichia coli* cultured in the presence of $H_2{}^{35}SO_4$ (15), in which case the specific activity was about 50 Ci/mmol. Virus labeled with [^{35}S]methionine was purified from radioactively labeled, infected ascites cells by the procedure of Kerr and Martin (13).

Electrophoresis on Polyacrylamide Gels was performed with a discontinuous buffer (16) and slab gels (10 cm × 16 cm × 1 mm) (17) with slots for simultaneous electrophoresis of 12 samples in parallel. Oocyte samples were homogenized in 15 mM Tris·HCl (pH 6.8) containing 150 μg/ml of phenylmethylsulphonyl fluoride. Ascites cells for analysis were suspended in the same buffer. Aliquots of such suspensions or homogenates, not exceeding half an oocyte per gel slot, were dissolved by boiling for 10 min with at least twice their volume of electrophoresis sample buffer [0.05 M Tris·HCl–1% sodium dodecyl sulphate–10 mM dithiothreitol–10% glycerol–0.001% bromphenol blue (pH 6.8)]. Electrophoresis was performed in gels containing 15% acrylamide and 0.09% bisacrylamide until the front, marked by bromphenol blue, had reached the end of the gel (about 15 hr at 30 V). Gels were dried under reduced pressure and bands were located by autoradiography on Kodirex x-ray film (Kodak).

Peptide Analysis. Proteins were eluted from specific regions of dried gels by suspension of finely chopped gel fragments in 0.5% dodecyl sulfate. Gel fragments were removed by filtration. Detergent was removed by repeated precipitation with 20% trichloroacetic acid and solution in 1 N NaOH. Lyophilized precipitated proteins were oxidized with performic acid (200 μl/50-μg sample) for 1 hr at 0°, then lyophilized twice. Oxidized proteins were digested with 10 μg of TPCK-treated trypsin (Worthington) in 100 μl of triethylamine carbonate (pH 9.0) for 1 hr at 37°, followed by addition of a further 10 μg of trypsin and 3 hr of incubation. Digests were lyophilized twice to remove triethylamine carbonate, suspended in electrophoresis buffer, and applied to 20 × 40 cm Polygram CEL 300 cellulose-coated thin-layer sheets for electrophoresis at pH 3.5 (5% acetic acid–0.5% pyridine) for 2 hr at 1 kV (25 V/cm). After location of peptides by autoradiography, the cellulose layer was divided into zones, scraped off, and chromatographed on cellulose thin layers in butanol–pyridine–acetic acid–water 5:1:4:4 (18).

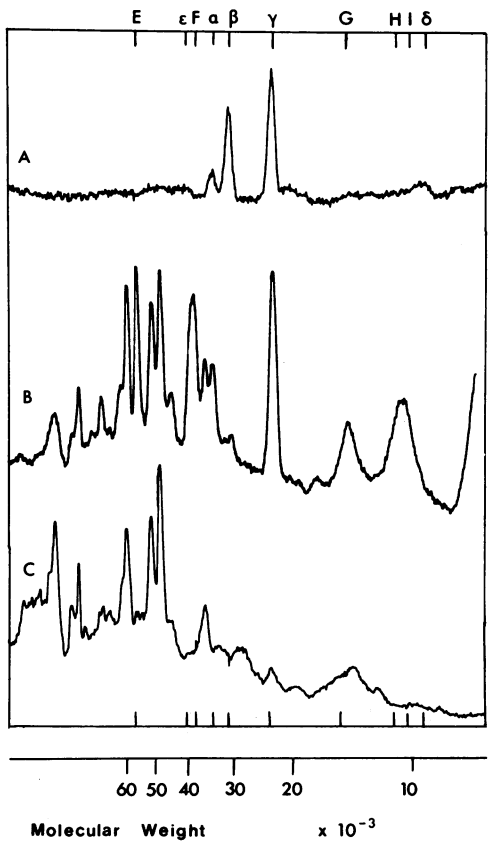

FIG. 1. Electrophoretic separation of EMC-specific polypeptides from ascites cells. (*A*) Purified EMC virions labeled with [^{35}S]methionine. (*B*) Ascites cells labeled with [^{35}S]methionine (100 μCi/ml) 3–7 hr after infection with 100 plaque-forming units (PFU)/cell of EMC virus in the presence of 5 μg/ml of actinomycin D. (*C*) Uninfected ascites cells labeled 3 hr with [^{35}S]methionine (100 μCi/ml) in the presence of 5 μg/ml of actinomycin D. Samples were prepared (*Methods*) and electrophoresed in 15% gels. Microdensitometer tracings of an autoradiograph of the dried gel are shown. The predicted positions of the EMC-specific proteins from infected HeLa cells (4) are indicated [nomenclature according to Butterworth *et al.* (4)].

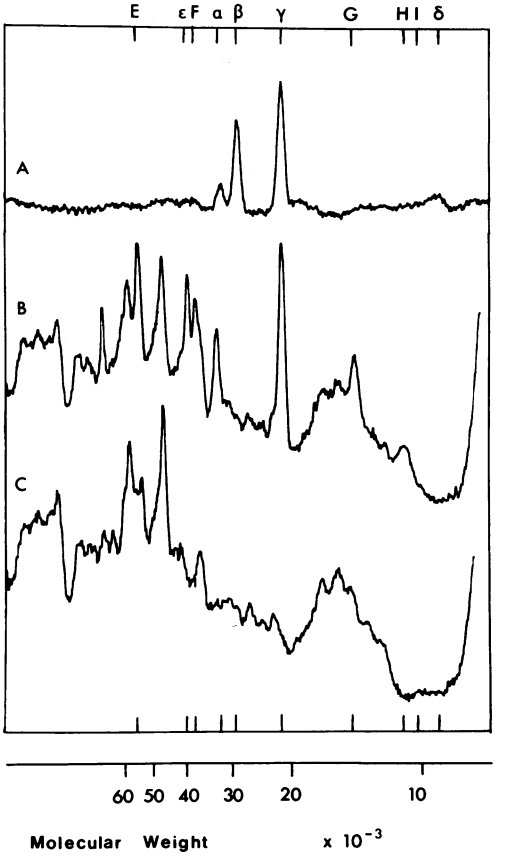

FIG. 2. Effect of EMC RNA injection on polypeptides synthesized by oocytes. (*A*) Purified EMC virions labeled with [^{35}S]methionine. (*B*) Oocytes injected with 50 nl of EMC RNA (2 mg/ml) and incubated in [^{35}S]methionine (250 μCi/ml) for 19 hr after injection. (*C*) Oocytes injected with saline and incubated in [^{35}S]methionine for 19 hr after injection. Electrophoresis conditions were as in Fig. 1. Microdensitometer tracings of an autoradiograph of the dried gel are shown. The predicted positions of the EMC-specific proteins from infected HeLa cells (4) are indicated.

RESULTS

EMC-specific proteins in infected ascites cells

Butterworth *et al.* (4) have described the EMC-specific proteins in infected HeLa cells. In the work described here, infected ascites cells have been used as standards for EMC-specific proteins. We have only investigated the stable proteins labeled in long pulses. Fig. 1 shows that the series of proteins synthesized in ascites cells after EMC infection closely resembles the series described by Butterworth *et al.* Although not shown in Fig. 1, the material of 40,000 daltons has been clearly resolved into two bands, corresponding to ϵ and *F* of Butterworth's nomenclature, but we have not been able to resolve the material of 12,000 daltons into two components corresponding to H and I. There is very little labeled material corresponding to β and δ, but these proteins are (4) cleavage products of ϵ, which is present. Differences in the distribution of radioactivity between the various bands from that observed by Butterworth *et al.* are expected because of the use of different labeled precursors. Identification of the virus-specific peaks in infected ascites cells was confirmed by electrophoretic comparison with labeled polypeptides from purified virions (Fig. 1*A*). Therefore, the nomenclature used by Butterworth *et al.* will be adopted here for describing the EMC-specific proteins in infected ascites cells.

Polypeptides synthesized in oocytes after injection of EMC RNA

About 50 nl of EMC RNA at 2 mg/ml in oocyte injection medium (2) was injected into each of a group of oocytes. Similar oocytes from the same frog were injected with 50 nl of injection medium without RNA. Groups of injected oocytes were incubated in [^{35}S]methionine (150–350 μCi/ml) in incubation medium (2) for up to 19 hr after injection. Oocytes were homogenized, boiled with dodecyl sulfate, and electrophoresed in polyacrylamide gels. Infected and uninfected labeled ascites cells, together with labeled virions, were boiled with dodecyl sulfate and electrophoresed in adjacent slots of the same gel for comparison. A homogenate of unlabeled oocytes was added to the ascites cell samples to balance the amounts of protein added to each slot of the gel.

As shown in Fig. 2, injection of EMC RNA into oocytes caused the appearance of a series of bands with identical mobilities to the series found in infected ascites cells. Furthermore, the proportion of radioactivity in each of the bands was similar in injected oocytes and infected ascites cells. This profile was highly reproducible and has been obtained from 32 groups of oocytes from six frogs with three different preparations of EMC RNA.

For each of the polypeptides that appeared in ascites cells after infection, a polypeptide of similar mobility and, therefore, molecular weight appeared in oocytes after injection of EMC RNA (Table 1). The possibility that these polypeptides contained identical peptide sequences was examined.

Analysis of peptides from proteins synthesized after injection of EMC RNA

The polypeptides from injected oocytes and infected ascites cells labeled with [^{35}S]methionine were separated on a preparative gel. Bands were located by autoradiography, eluted, and digested with trypsin. Peptides were separated first by electrophoresis, then by chromatography. Fig. 3 shows an electrophoretic comparison of the peptides from the γ band of infected ascites cells and from the major band that comigrates with γ from oocytes injected with EMC RNA.

The conspicuous similarity in profile was confirmed by dividing the cellulose layer into the zones indicated in Fig. 3, eluting the peptides from each fraction, and chromatographing them in a second dimension. Cross-contamination of samples was avoided by excluding the sample boundaries of both the gel and the thin layer from further analysis. Fig. 4 shows that the EMC γ protein and the protein from injected oocytes that comigrates with γ contain many peptides that behave identically upon both electrophoresis and chromatography.

A parallel analysis of peptides was performed on the same region of gels loaded with uninfected ascites cells and oocytes that had been injected with saline. Due to the small amounts of radioactivity in this region of the gel, only faint bands were observed in the peptide analysis. None of the major matching peptides shown in Fig. 3 could be detected in either uninfected ascites cells or mock-injected oocytes. We conclude that the γ protein of EMC is synthesized by oocytes after injection of EMC RNA.

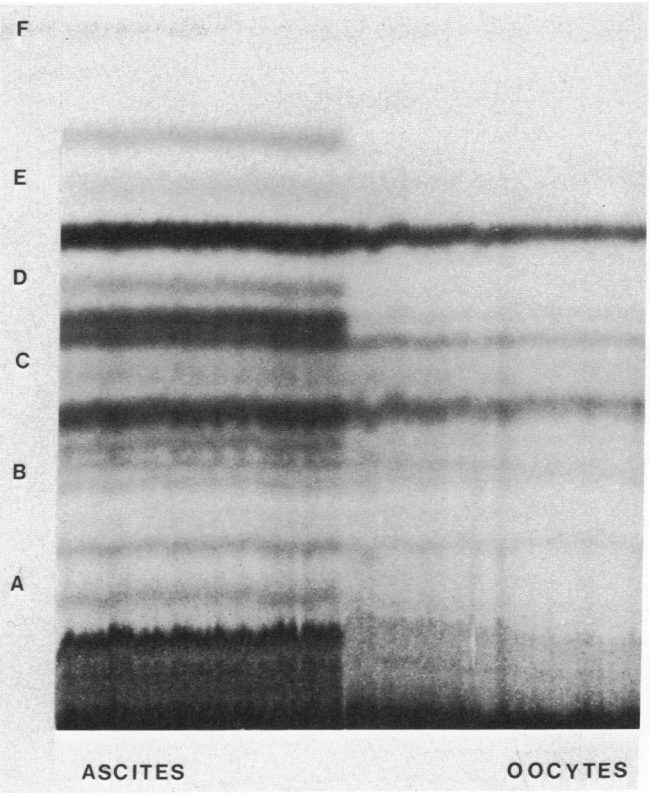

FIG. 3. Electrophoretic comparison of methionine-containing tryptic peptides of the putative γ protein from injected oocytes and infected ascites cells. ^{35}S-labeled γ from infected ascites cells, and the protein from injected oocytes that comigrates with γ, were prepared by polyacrylamide gel electrophoresis as in Figs. 1 and 2. They were digested with trypsin and the products were electrophoresed at pH 3.5. An autoradiograph of the dried thin-layer chromatogram is shown. Bands that are present in only one sample and not the other may be due to cellular proteins contaminating the γ region of the polyacrylamide gel. The lettered regions were collected for a further chromatographic comparison (see Fig. 4).

TABLE 1. *The range of stable EMC-specific proteins detected in infected ascites cells and in oocytes injected with EMC RNA*

Protein*	Apparent* molecular weight	Present in virion	Detected in ascites cells	Detected in oocytes
E	56,000	−	+	+
ϵ	40,000	+	+	+
F	38,000	−	+	+ ?†
α	34,000	+	+	+
β	30,000	+	−	−
γ	23,000	+	+	+
G	16,000	−	+	+
H	12,000	−	+	+‡
I	11,000	−		
δ	9,000	+	−	−

* Nomenclature and apparent molecular weights are cited from Butterworth *et al.* (4).

† The detection of F in oocytes is uncertain because of a contaminating oocyte protein of similar mobility.

‡ The material in this region was not resolved into two peaks corresponding to H and I.

A similar peptide analysis was performed on the polypeptides that comigrated with G (16,000 daltons) and H + I (not resolved—12,000 daltons). In the case of G analysis was by electrophoresis only, but in the case of (H + I) both electrophoresis and chromatography were used. In both cases matching peptides were observed, at least seven in G and at least 16 in (H + I). Therefore, proteins synthesized in oocytes in response to injection of EMC RNA resemble EMC-specific proteins both in size and in the sequence of their methionine-containing peptides.

DISCUSSION

Evidence that EMC RNA is translated by Xenopus oocytes

Injection of EMC RNA into oocytes induced the appearance of at least six new labeled polypeptides. All of these comigrated on gels with bands that appeared in ascites cells after EMC

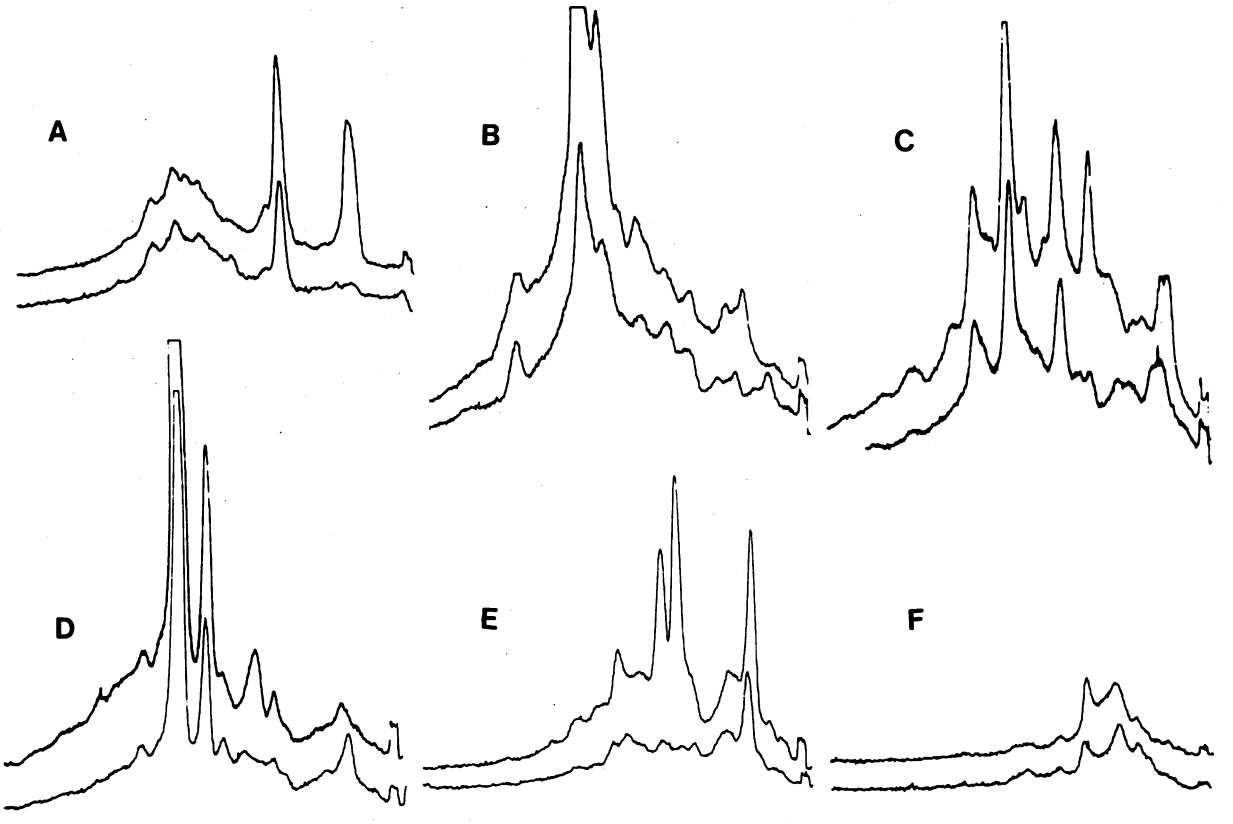

FIG. 4. Chromatographic comparison of peptides from putative γ. The peptide fractions indicated in Fig. 3 were prepared and chromatographed as described in *Methods*. Microdensitometer tracings of autoradiographs of dried thin-layer chromatograms are shown. *Letters* indicate the fraction of the electrophoretic separation that was chromatographed. For each pair of tracings, the *upper trace* is derived from EMC γ protein from infected ascites cells and the *lower trace* from oocytes injected with EMC RNA.

infection. Two of these bands comigrated with virion proteins ($\gamma + \alpha$). A third virion protein (β) was not always observed in either injected oocytes or infected ascites cells, but Butterworth *et al.* (15) showed that this was a cleavage product of another virus-specific protein ϵ. A band of the same molecular weight as ϵ (40,000) was routinely observed.

Therefore, all of the new detectable polypeptides synthesized by oocytes in response to injection of EMC RNA were the same size as EMC-specific polypeptides.

Comparison of the methionine-containing tryptic peptides from three of the induced polypeptides in injected oocytes with γ, G, and (H + I) from infected ascites cells confirmed that EMC-specific proteins are synthesized in oocytes after injection of EMC RNA. This fact is surprising since the temperature used for oocyte incubations (18–20°) is below the minimum temperature at which EMC translation is detectable in the ascites cell-free system (19), suggesting that the inability of the ascites system to translate EMC RNA at low temperature is not due to condensed secondary structure of the RNA. Furthermore, in contrast with infected HeLa cells or ascites cells, virus-specific protein synthesis in oocytes was easily recognized without suppression of endogenous protein synthesis by actinomycin D.

The extent of translation of EMC RNA in oocytes

Butterworth *et al.* (4) suggested that the stable EMC-specific proteins are formed by progressive cleavage of larger precursors. The EMC genome appears to contain only a single initiation site, so that internal initiation within the message does not occur‡. In the scheme proposed by Butterworth *et al.* (4), the EMC-specific proteins whose synthesis we have demonstrated in oocytes are distributed throughout the genome and are not clustered in a particular portion. This fact, together with the observation that injected oocytes synthesized proteins of the same size as all of the proteins that could be detected in ascites cells during a normal productive infection, strongly suggests that oocytes translated the entire EMC genome.

We have not directly demonstrated cleavage from large precursors, but the fact that all the products in frog oocytes were the same size as those in HeLa cells where the cleavage is known to occur (4) strongly suggests that post-translational cleavage occurred in oocytes. The cleavage mechanism could be specified by the host cell or, less likely, by the virus. The cleavage mechanism must clearly occur very widely.

Most of the animal mRNAs that have been tested in oocytes have proven to be active in directing protein synthesis. The only exception, to our knowledge, among those properly tested is RNA from the RNA tumor viruses. Injected oocytes offer several important advantages for the study of translation over virus-infected mammalian cells, and cell-free ex-

‡ Smith, A. E. (1972) *Eur. J. Biochem.*, submitted.

tracts derived from them. Viral products are recognizable in oocytes without the necessity of suppression of endogenous synthesis by the addition of drugs, such as actinomycin D. Conditions for translation can easily be varied by injection of other components into the oocytes. In comparision with the ascites cell-free system, translation in oocytes is more complete and efficient. The stability of the functional EMC RNA in oocytes is remarkable, translation continuing for more than two days. In addition to correct translation, the oocytes are capable of completing the post-translational processing that generates functional EMC proteins in their normal host cells.

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