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Translation of Rabbit Haemoglobin mRNA in Oocytes of the Queensland Cane Toad, *Bufo marinus*

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Abstract

Rabbit haemoglobin mRNA is translated in oocytes of the toad, *B. marinus*. The product was identified by Sephadex chromatography, tryptic peptide analysis and CM-cellulose chromatography using authentic rabbit haemoglobin as a marker. Oocytes of this species provide a suitable alternative to those of *Xenopus laevis* for the detection of mRNA.

Introduction

Gurdon first demonstrated that oocytes of the South African clawed toad, *Xenopus laevis*, synthesized rabbit haemoglobin when injected with rabbit haemoglobin mRNA (Gurdon *et al.* 1971). Subsequently, this system has proven a useful and sensitive method for translating a number of eukaryote and viral mRNAs (Lane *et al.* 1971; Berns *et al.* 1972; Laskey *et al.* 1972). However, to date only oocytes from *X. laevis* and fertilized eggs from *Pleurodeles watlii* (Brachet *et al.* 1973) have been used.

We have found that oocytes of the Queensland cane toad, *Bufo marinus*, will translate injected rabbit haemoglobin mRNA with an efficiency comparable with that of oocytes of *X. laevis*. We report this finding because of the possible usefulness of this technique to workers interested in the detection of mRNA in this manner. Moreover, *Bufo* species, unlike *X. laevis*, are found in all continents of the world, do not require entirely aquatic conditions (as does *X. laevis*) and are easily maintained in the laboratory.

Materials and Methods

Toads

Female *B. marinus* were obtained from the Apex Club, Innisfail, Qld. The toads were kept on damp sand.

Microinjection

Micropipettes were made from Temmo plain capillary tubes of diameter 1·45–1·65 mm (Jintan Temmo Co. Ltd, Tokyo). Pipettes were pulled out manually to 100 μm and then pulled out further to 10 μm in a micropipette maker. They were calibrated to deliver a volume of about 50 nl. All glassware was alkali-washed to remove contaminating ribonucleases. Microinjection syringes were filled with water rather than paraffin oil as used by Gurdon and coworkers.

Measurement of Protein Synthesis

Protein synthesis was measured by incubating oocytes in Barth's medium (Gurdon 1967) containing the appropriate radioactive amino acid. Oocytes were incubated at 22°C, then homogenized in 0·052M tris–glycine buffer (pH 8·9) containing unlabelled amino acid (0·5%). Protein was precipi-

tated with 5% trichloracetic acid containing unlabelled amino acid (0·5%). The precipitate was treated with alkali then reprecipitated and twice washed with trichloracetic acid, ethanol and ether and finally dissolved overnight in 0·1 ml of NCS tissue solubilizer (Amersham/Searle Corp., Ill., U.S.A.). Scintillation fluid (2·0 ml) containing 0·3% 2,5-diphenyloxazole and 0·03% 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene was added and the samples counted in a Packard Tri-Carb spectrometer. Radiochemicals were from Schwarz Mann, Orangeburg, N.Y., or the Radiochemical Centre, Amersham, England.

Gel Filtration

Columns of Sephadex G100 (1 by 140 cm) were prepared by the method of Andrews (1964). Columns were equilibrated with 0·05M tris–glycine buffer (pH 8·9) and run at 2–4°C. The flow rate was 10 ml/h and 1-ml fractions were collected.

Tryptic Peptide Analysis

Samples from which haem had been removed were freeze-dried and the lyophilized material taken up in 4–5 ml of 0·2M *N*-ethylmorpholine–acetate buffer (pH 8·3); 1 mg of trypsin (Worthington) was then added and the mixture incubated at 37°C for 4 h. The solution was then evaporated to dryness and taken up in acetate buffer (pH 3·1), and the peptides analysed on Technicon Chromobeads (type P) cation ion-exchange resin. Peptides were eluted with a pyridine–acetate concave gradient (Schroeder *et al.* 1962).

CM-cellulose Chromatography

Samples from which haem had been removed were taken up in 2 ml of 0·2M formic acid–0·02M pyridine buffer, dialysed for 2 h against the same buffer and centrifuged at 35 000 *g* for 60 min. The supernatant was then loaded onto a Whatman carboxymethyl 32 cellulose column and eluted with a pyridine–formic acid gradient (Rabinowitz and Fisher 1964).

Preparation of Rabbit Haemoglobin mRNA

Purified 9 S RNA was prepared from ribonucleoprotein particles and was the generous gift of Dr D. J. Kemp of this Department.

Results and Discussion

Effect of Microinjection on Endogenous Protein Synthesis

Oocytes were obtained by dissection of female toads; prior injection with pituitary hormone was unnecessary. The largest oocytes were 1·0–1·5 mm in diameter (similar in size to those of *X. laevis*) and were used in all microinjection experiments. Immediately after dissection the oocytes were separated, washed once in Barth's medium and maintained in this medium. Preliminary experiments showed that a variety of radioactive amino acids, including leucine, isoleucine, valine, phenylalanine, cystine and histidine, were readily incorporated from the medium into endogenous protein and that such incorporation proceeded linearly for 40–50 h.

Control experiments showed that injection of up to 100 nl of the buffer used to dissolve mRNA (0·01M tris–HCl, pH 7·6) had no effect on this endogenous protein synthesis for at least 24 h.

Injection of Rabbit Haemoglobin mRNA

In order to test whether oocytes of *B. marinus* were capable of translating a foreign mRNA species, experiments were carried out in which purified 9 S rabbit haemoglobin mRNA was injected.

Separate batches of 30 oocytes were each injected with 50 nl of either 0·01M tris–HCl buffer, pH 7·6, or haemoglobin mRNA in this buffer (at about 300 μg/ml).

They were then incubated for 24 h at 22°C with 50 μl of Barth's medium (Gurdon 1967) containing 50 μCi of L-[2,5-^{3}H]histidine (specific activity 58 Ci/mmol). Oocytes were then homogenized in 1 ml of 0·05M tris–glycine buffer, pH 8·9, containing 0·5% (w/v) L-histidine. Rabbit haemoglobin (10 mg) was added and the preparation centrifuged at 75 000 *g* for 45 min, then the soluble fraction was applied to a Sephadex G100 column. A large peak of radioactivity coincident with marker haemoglobin was observed when rabbit haemoglobin mRNA was injected (Fig. 1*b*), but not when buffer alone was injected (Fig. 1*a*). The identity of the other two major radioactive peaks (eluting between 125 and 150 ml) are unknown, but could be the product of non-haemoglobin mRNA species present in the 9 S RNA preparation.

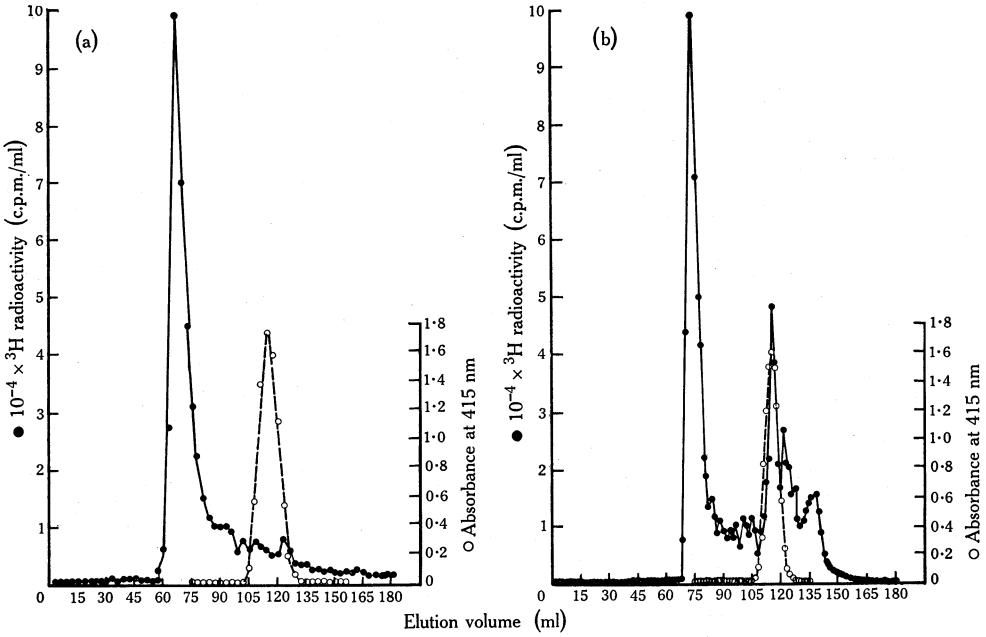

Fig. 1. Sephadex analysis of oocytes injected with either (*a*) 0·01M tris–HCl buffer, pH 7·6, or (*b*) haemoglobin mRNA in this buffer. Total radioactivity (●) was determined in each fraction by liquid scintillation in Bray's scintillation fluid. Marker haemoglobin was determined by measuring absorbance at 415 nm (○).

Identification of Presumptive Rabbit Haemoglobin

To confirm that rabbit haemoglobin mRNA had been translated in the oocytes, material from the haemoglobin region in Fig. 1(*b*) was pooled and additional haemoglobin carrier (35 mg) added. Haem was removed by treatment with acetone–HCl containing β-mercaptoethanol (Lane *et al.* 1971). The preparation was then dialysed against water, freeze-dried and digested with trypsin. The resulting tryptic peptides were analysed by cation-exchange chromatography. Fig. 2 shows that there were at least seven radioactive tryptic peptides coincident with peptides derived from digestion of authentic haemoglobin. Theoretically, digestion of histidine-labelled rabbit haemoglobin should yield 12 radioactive tryptic peptides, but in practice a smaller number are often observed (see Lane *et al.* 1971). The identity of the two large peaks of radioactivity eluting between fractions 50 and 60 is unknown, but could possibly arise from

contaminating non-haemoglobin proteins. This result again casts some doubt on the purity of the mRNA preparation used. Nevertheless the results provide strong evidence that the material derived from the Sephadex peak contains radioactive haemoglobin.

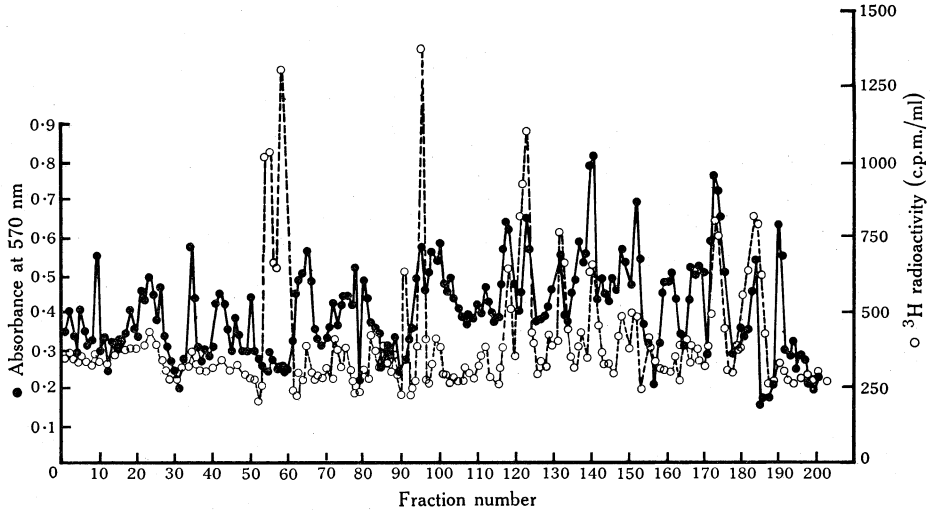

Fig. 2. Tryptic peptide analysis of the pooled material from the haemoglobin region of the Sephadex G100 column. Radioactivity (○) was determined by drying samples on Whatman GF/A glass fibre filters and counting by liquid scintillation spectrometry. Peptide material was determined by the absorbance at 570 nm (●) following the ninhydrin reaction.

Further evidence that the haemoglobin mRNA had been translated was obtained by CM-cellulose chromatography. A portion of the material derived from the presumptive haemoglobin peak of Fig. 1(*b*) was converted to globin chains and analysed on a CM-cellulose column as outlined in the Methods section. It was found that radioactivity co-eluted with both α- and β-globin marker chains (Fig. 3).

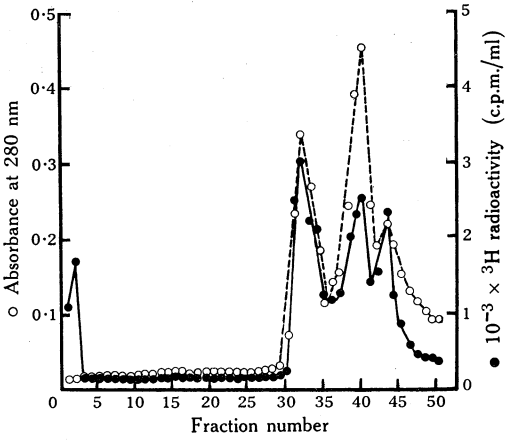

Fig. 3. CM-cellulose chromatography of material from the haemoglobin region of the Sephadex G100 column.

The identity of the third peak of material that absorbs at 280 nm and is associated with radioactivity is unknown, but could possibly be haemoglobin that has not had

the haem removed, as suggested by Gurdon *et al.* (1971), or may possibly be δ-haemoglobin chains.

It was interesting to note that translation of rabbit mRNA in *X. laevis* oocytes resulted in an excess synthesis of β- over α-chains (Lane *et al.* 1971) whereas the reverse was found to be true in the present work. A similar α-chain excess is also observed when haemoglobin mRNA preparations similar to those used in this study are translated in a chick feather lysate (G. A. Partington and D. J. Kemp, personal communication).

This work, together with that of Gurdon *et al.* (1971) and Brachet *et al.* (1973), suggests that many species of amphibian oocytes may be suitable for translating mRNA. The availability and convenience of handling of *Bufo* species make them potentially attractive to workers interested in the detection of mRNA in this manner.

Acknowledgments

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