9 S HAEMOGLOBIN MESSENGER RNA FROM RETICULOCYTES AND ITS ASSAY IN LIVING FROG CELLS

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Introduction

Mammalian reticulocytes are cells which have undergone physiological enucleation; they may be expelled into the peripheral circulation under condition of acute anaemia. However, these cells are still able to synthesize protein (95 % of which is haemoglobin) for several hours. This observation suggests that there is a stable haemoglobin messenger RNA in the cytoplasm of the reticulocyte. Over the years several different research groups have attempted to isolate the messenger RNA for haemoglobin : for the reticulocyte system presents a favourable opportunity, perhaps the most favourable, for isolating a messenger RNA corresponding to a well characterized protein.

In Chantrenne's laboratory, Marbaix and Burny (1964) (1), Burny and Marbaix (1965) (2), Marbaix, Burny, Huez and Chantrenne (1966) (3), Chantrenne, Burny and Marbaix (1967) (4) and Huez, Burny, Marbaix and Lebleu (1967)(5) were able to isolate and characterize a 9 s RNA fraction.

from rabbit reticulocyte polyribosomes which had many of the properties expected of the messenger RNA for globin chains.

In 1969, Lockard and Lingrel (6) showed that a 9 s RNA fraction obtained from mouse reticulocyte polyribosomes by a method similar to that used in Chantrenne's laboratory directs the synthesis of mouse globin β chains in a rabbit reticulocyte system. Further experiments showed that this fraction also contained the message for the α chain (7). It was thus proved that the 9 s RNA fraction from reticulocyte polyribosomes contains the messages for both globin chains.

In this communication a method for preparing highly purified 9 s RNA is described (8) : it is an improvement on that described by Huez et al., (5). The activity of the 9 s RNA is assayed using living frog cells, namely *Xenopus* oocytes ; the advantages of this new assay system for messenger RNA are discussed.

Preparation of 9 s RNA from rabbit reticulocytes

Rabbits were made anaemic and were bled as described previously (1). Blood cells were collected by low-speed centrifugation and washed twice in an isotonic saline medium. Differential lysis of reticulocytes and leucocytes was brought about by adding two volumes of cold hypotonic saline, shaking for two minutes and then re-establishing isotonicity by adding 0.2 volumes of 1.5 M NaCl. The lysis of leucocytes was thus avoided. Intact cells and stroma were eliminated by low-speed centrifugation. The stroma-free lysate was layered on 5 ml cushions of 30 % sucrose made in 5 mM Tris-HCl, 20 mM KCl, 1 mM Mg acetate, pH 7.6 and the tubes were centrifuged for two hours at 30,000 rpm. in the type 30 rotor of the Spinco ultracentrifuge.

The polyribosomes pellets were then resuspended in 6.6 mM phosphate buffer containing 33 mM EDTA (pH = 7.0) and 1 ml fractions of the resulting suspension (10 mg/ml) were layered on 15-30 % linear sucrose gradients made in 0.01 M sodium phosphate pH 7.0. After a 40 h centrifugation at 25,000 rpm and 4°C in the SW 27 Spinco rotor, fractions were collected and analyzed for absorbance at 260 nm.

Fig.1 shows the result of such a centrifugation. Thus treatment with 33 mM EDTA causes complete disaggregation of the polyribosomal structure : the ribosomal subunits fall apart, and the 5 s RNA and the 9 s messenger RNA are released as ribonucleoproteins sedimenting at 8 and 14 s respectively (8). As shown in Fig.1 the centrifugation step was long enough to cause the ribosomal subunits to reach the bottom of the tube, thereby achieving good resolution in the 14 s mRNP region of the gradient.

The sucrose gradient fractions which contain the mRNP were pooled and the solution was made 0.4 M in NaCl and 1 % (w/v) in sodium dodecylsulphate (SDS). Two volumes of ethanol were added and RNA and protein were allowed to precipitate by standing overnight at -20°C. The precipitate was collected by low-speed centrifugation and was dissolved in a solution of 1 % (w/v) SDS (1 ml of solution per 100-200 µg of RNA). 1 ml portions of the resulting RNA solution were layered on 10-20 % sucrose gradients made in 10 mM Tris-HCl, pH 7.4. After centrifugation (using a Spinco SW 27 rotor) for 40 h at 25,000 rpm and at 4°C, fractions were collected and analyzed for their absorbance at 260 nm. Fig.2 shows the result of such a centrifugation step. The 9 s RNA is now almost pure and is devoid of any contaminating 5 s RNA. Fractions corresponding to the pure 9 s RNA were pooled and were made 0.4 M in NaCl. Two volumes of ethanol were added and the RNA was allowed to precipitate at -20°C overnight. The 9 s RNA was then collected by centrifugation, dissolved at a concentration of 100 µg/ml, in 10 mM Tris-HCl buffer pH 7.4 and residual proteins were removed by two extractions with a mixture of chloroform and isoamyl alcohol (24:1 v/v). The RNA solution was then dialysed for 15 h against two changes of 1,000 volumes of double-distilled water, and was then lyophilized.

Starting with 10 rabbits (having a reticulocytosis of from 50 to 90 %) one can obtain 1 mg of purified 9 s m-RNA.

The assay of globin messenger RNA in frog oocytes

A sensitive assay system for messenger RNA has recently been developed by Lane, Marbaix and Gurdon (9) and by

Gurdon et al. (10). The following description illustrates the use of this system for the assay of haemoglobin m-RNA.

In the experiments described here, oocytes were taken from frogs (Xenopus laevis) which had been induced by hormone treatment to ovulate between two and four weeks previously. This was done to ensure that the larger oocytes were actively growing. Oocytes were injected dry and without removal of follicle cells (11). Micropipettes of 10-15 μ diameter were calibrated to deliver a volume of 50-70mμl the actual amount being kept constant in each related series of experiments. All samples to be injected were taken up in or dialysed into the following injection medium: 88 mM NaCl, 1,0 mM KCl, 15 mM Tris-HCl, pH 7.6. Injected cells were incubated at 19°C in the saline medium described by Gurdon (11). Oocytes were labelled by incubation in this culture medium containing from 0.1 to 1 mCi/ml of ^{3}H-histidine (30-50 Ci/mM). At the end of the incubation cells were frozen.

Using these techniques, the following experiments were carried out to test the effects of introducing 9 s reticulocyte RNA into living oocytes.

30 oocytes were each injected with the standard solution containing haemin (0.5 mg/ml) and 9 s RNA (700 μg/ml) and were incubated for 6 h in a ^{3}H-histidine-containing medium. Another batch of 30 cells were treated similarly except that the injectate contained no 9 s RNA. After freezing, cells were homogenized in a 0.0522 M glycine-0.0522 M Tris - 0.1 % (w/v) histidine buffer (pH 8.9) containing 5 mg/ml of commercial rabbit haemoglobin. Homogenates were centrifuged at 75,000 g for 30 minutes at 4°C and the resulting supernatant was applied directly to a 140 x 1 cm G100 Sephadex column equilibrated with 0.0522 M glycine - 0.0522 M Tris buffer (pH 8.9). Fig.3 shows the elution profiles obtained from the samples described. The presence of 9 s RNA in the injectate is linked to the appearance of a peak of radioactivity with an elution profile identical to that of added marker rabbit haemoglobin (9).

Material from the haemoglobin region of the Sephadex profile was mixed with the appropriate solutions and loaded onto a polyacrylamide gel. After electrophoresis using the method of Moss and Ingram (1968) (12), a sharp peak of radioactive material was found to have moved with

the marker haemoglobin (Fig.4). In controls lacking 9 s RNA material taken from the optical density peak of haemoglobin showed no preferential migration of radioactivity with marker haemoglobin (9). Thus the molecules whose synthesis is caused by the presence of 9 s RNA are, under these conditions, indistinguishable from haemoglobin, in overall charge distribution as well as in size.

If the material whose synthesis within the frog oocyte is directed by rabbit 9 s RNA is really haemoglobin, it should be possible to show that this material contains α and β globin chains. Thus the haemoglobin region of the Sephadex elution profile was pooled, mixed with more marker rabbit haemoglobin and treated with acid acetone to yield a precipitate of globin. If this protein preparation is then analysed by chromatography on a CM-cellulose column (after Dintzis,1961) (13), it is found that the presence of 9 s RNA in the injection medium is linked to the appearance of radioactive material that co-chromatographs with marker rabbit globin chains (Fig.5). If 9 s RNA is omitted from the injection mixture, no material which preferentially migrates with globin chains can be detected (9).

Definite identification of the polypeptides peculiar to 9 s RNA injected oocytes was provided by an analysis of the tryptic peptides derived from this material. α and β chains, uniformly labelled with ^{14}C-histidine, were prepared by incubating rabbit reticulocytes with this aminoacid the separated chains were then mixed with oocyte-derived ^{3}H-histidine labelled α and β chains. These mixtures were digested with trypsin and the resulting α and β tryptic peptides were analyzed using a cation exchange resin column. Fig.6 shows the result of such an analysis for the tryptic peptides of the β chain. It is clearly seen that all the peptides from the two different sources coincide perfectly, both qualitatively and quantitatively (except for one peptide in the latter instance).

It is worth mentioning that neither 26 s , 18 s and 4+5 s RNA from the reticulocyte nor 9 s RNA from another tissue (mouse myeloma) cause the synthesis of globin in injected oocytes. Furthermore, the translation of 9 s RNA within the oocyte does not require added haemin : nor does this process require any reticulocyte-specific factors (10).

The globin synthesized resembles rabbit as opposed to frog globin, as shown by its chromatographic behaviour on CM-cellulose : the frog proteins elute after both rabbit chains. Moreover, the sequence of the β chain of Rana esculenta globin is known to be very different to that of the rabbit β chain (14). Although the sequence of the β chain of Xenopus globin is not known, one can, nonetheless, argue that it is likely to be quite different from that of the rabbit β chain. Consequently, Xenopus globin tryptic peptides would not be expected to coincide with tryptic peptides derived from rabbit globin.

Conclusions and Discussion

The results obtained confirm those of Lockard and Lingrel (1969) (6) who used an in vitro system to provide evidence that the 9 s RNA fraction from reticulocytes contained haemoglobin messenger activity. However, this work presents the most complete characterization of α and β globin chains synthesized, in a heterologous system, under the direction of 9 s messenger RNA.

A more interesting point is that the oocyte provides an excellent system for testing messenger RNA's (16). Firstly, the translation of the message occurs in a normal living cell and is, therefore, less likely to be affected by artefacts than it would be in a cell-free system. Secondly, injected oocytes are able to translate messages very efficiently, for long periods of time : for example, one can obtain, over a 24 h period, as much as 10^6 dpm of labelled Hb from 1 mμg of injected m-RNA ; furthermore, ribosomal RNA is not inhibitory to this process, for 9 s RNA mixed with more than 10 times as much 18 s RNA is still translated efficiently. Lastly, Xenopus oocytes appear to show very little species specificity with regard to the type of m-RNA which they can translate. Other types of m-RNA's have already been tested in this system, both 9 s RNA from mouse reticulocytes (15) and 9 s m-RNA from mouse myeloma cells (16) are readily translated.

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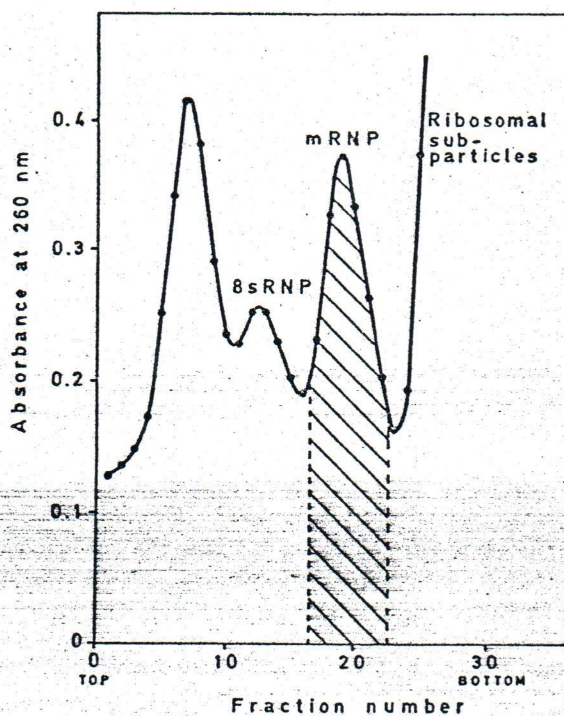

Fig. 1 - The figure shows the sedimentation pattern obtained by centrifuging a suspension of reticulocyte polyribosomes treated with 33 mM EDTA. The ribosomal subunits have reached the bottom of the tube. The 15-30 % linear sucrose gradients used were made up in 10 mM sodium phosphate buffer pH 7.0 : centrifugation was performed at 6°C for 40 h at 25,000 rpm. using a Spinco SW 27 rotor.

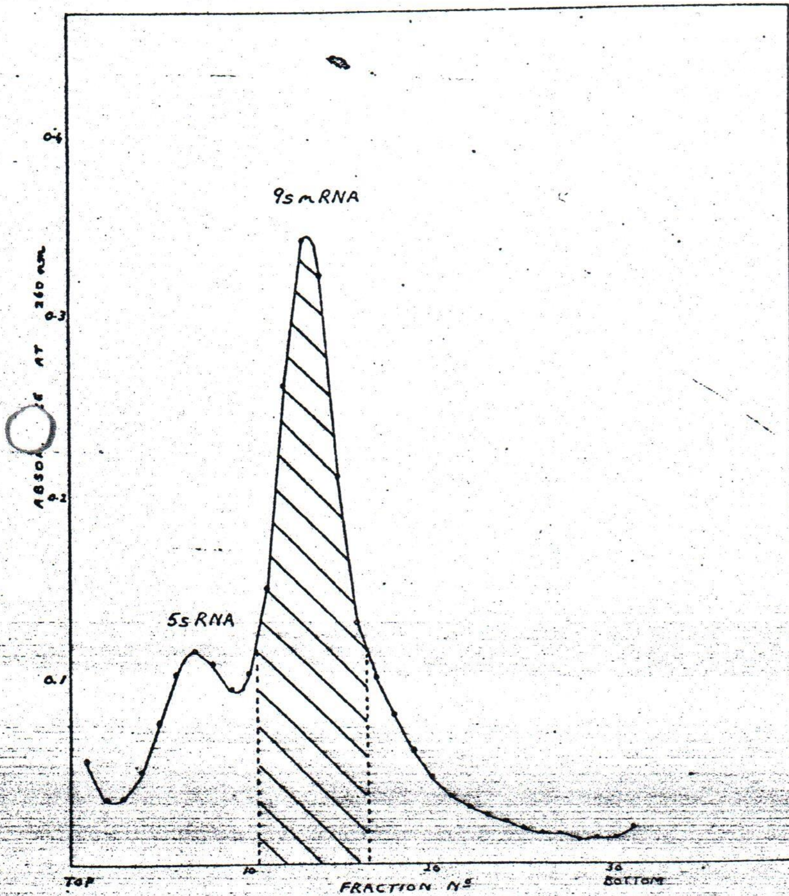

Fig. 2 - The figure shows the sedimentation pattern of the RNA extracted from the ribonucleoprotein material corresponding to the hatched area shown in Fig.1. The 10-20 % linear sucrose gradients used were made up in 5mM Tris-HCl pH 7.4 : centrifugation was performed at 4°C for 40 h at 24,000 rpm using the Spinco SW 25.1 rotor.

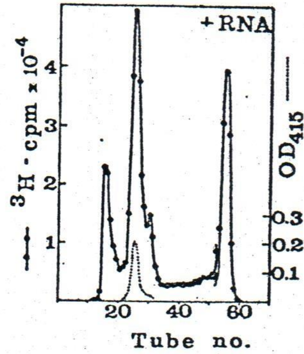

Fig. 3.a shows the Sephadex G 100 elution profile of supernatant material from oocytes which were injected with 9 s RNA (dissolved in a haemin-containing buffer at a concentration of 700 mg/ml) and cultured for 6 h in ^{3}H-histidine at 1 mCi/ml.

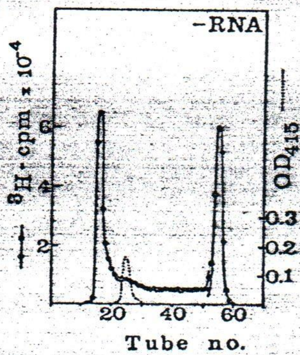

Fig. 3.b is identical except that the injectate contained no 9 s RNA. Cts/min refer to material from a single oocyte Open (O——O) refer to optical density at 415 nm and closed circles (●——●) refer to cts/min.

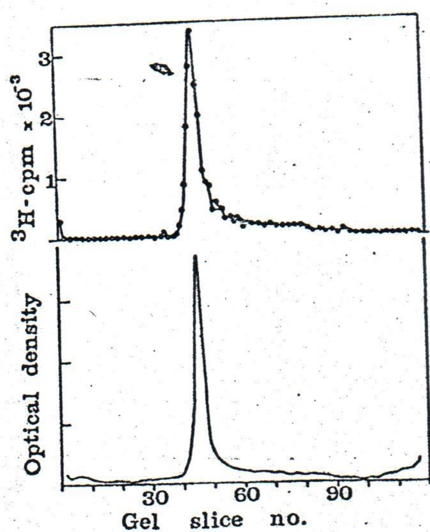

Fig. 4 - This figure shows the distribution pattern of material from the haemoglobin region of the Sephadex elution profile after gel electrophoresis at 5 mA/gel for 90 min.
The continuous line (———) refers to optical density at 415 nm and the closed circles (o———o) to cts/min.

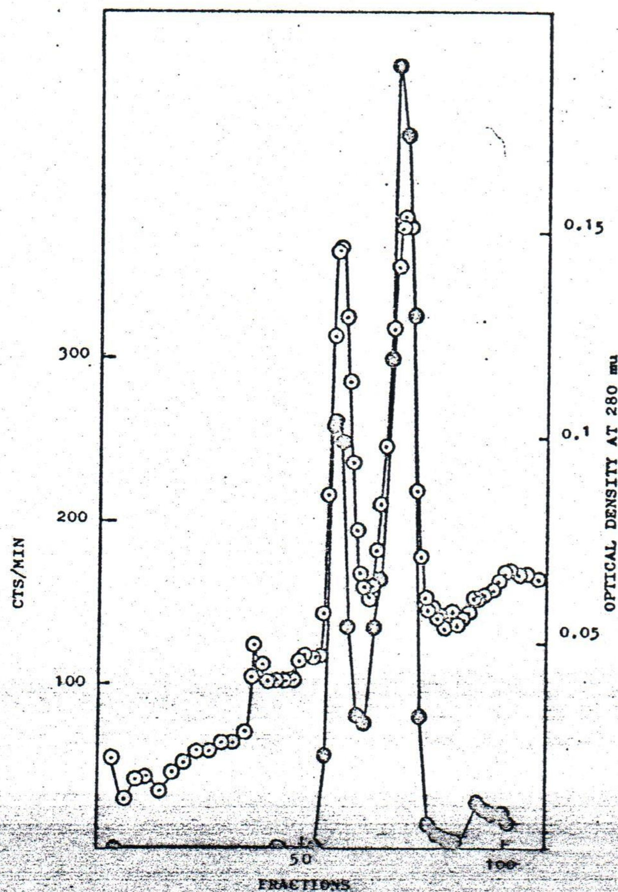

Fig. 5 – This figure shows carboxymethyl-cellulose chromatography of globin chains. Material from the haemoglobin region of the Sephadex elution profile was used to prepare globin ; further marker rabbit haemoglobin was added prior to this stage.
Open circles (O——O) refer to optical density at 280 nm and closed circles (●——●) refer to cts/min.

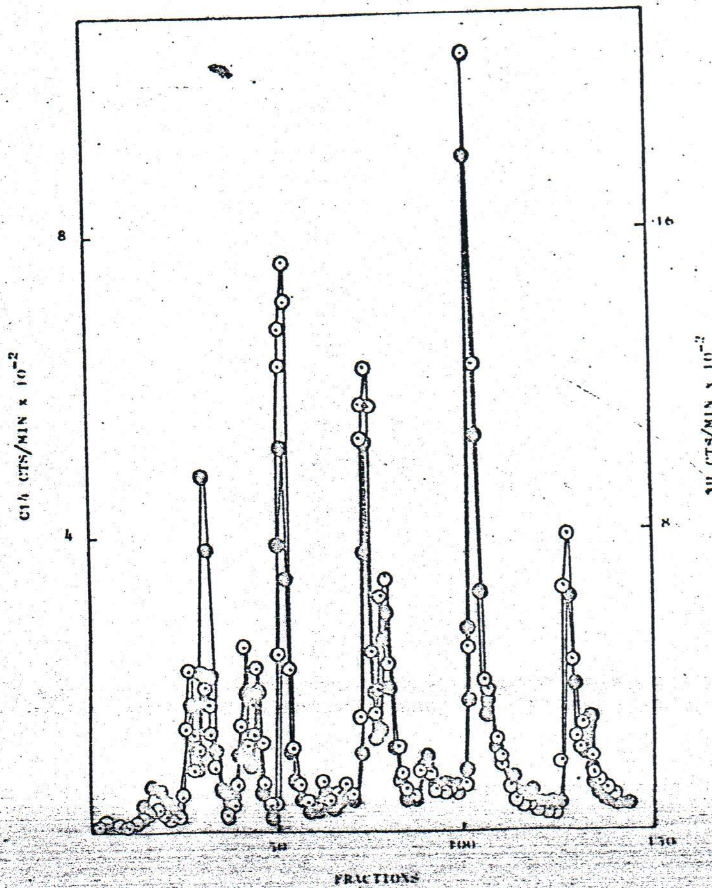

Fig. 6 - ^{14}C-His-β globin chains from rabbit reticulocytes were mixed with ^{3}H-His-β globin chains from oocytes and the resulting solution digested with trypsin. The peptide mixture was then analysed on a Technicon Chromobead (type P) cation exchange resin eluted with a formic acid-pyridine gradient, thereby yielding the pattern shown. Open circles (O——O) refer to ^{3}H cts/min and closed circles (●——●) refer to ^{14}C cts/min.