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II†. Further Characterization of the Products of Translation of Reticulocyte 9 s RNA

Oocytes of the frog *Xenopus laevis*, when injected with rabbit reticulocyte 9 s RNA dissolved in buffer, synthesize material resembling rabbit α- and β-globin chains. Oocytes injected with buffer alone contained no detectable amounts of such material. Oocyte-derived globin chains were found to be similar to rabbit reticulocyte globin chains as judged by carboxymethyl cellulose chromatography of the whole chains and by cation-exchange chromatography, paper chromatography and paper electrophoresis of tryptic peptides derived from the separated α- and β-chains.

It is concluded that no reticulocyte specific factors are required for the translation of rabbit reticulocyte 9 s RNA in frog oocytes and that the translational machinery of the frog cell will accept the messenger RNA from a totally different cell-type, from another species. These results confirm and extend those of Lane, Marbaix & Gurdon (1971).

It has recently been shown (Lane, Marbaix & Gurdon, 1971) that oocytes from the frog *Xenopus laevis*, when injected with 9 s RNA from rabbit reticulocytes, synthesize material resembling rabbit globin chains. The 9 s RNA was injected as a solution made up in a haemin-containing buffer: and oocytes injected with the haemin solution alone did not synthesize detectable amounts of globin-like material. The protein synthesized in the oocyte under the direction of 9 s RNA was found to be similar to rabbit haemoglobin as judged by gel filtration and gel electrophoresis; after acid/acetone treatment the protein was found to resemble rabbit globin in its chromatographic behaviour on carboxymethyl cellulose. Cation-exchange chromatography of histidine-labelled tryptic peptides from oocyte-derived, and reference rabbit reticulocyte haemoglobins demonstrated that there were no peptides present in the material from oocytes that were not also present in the material from reticulocytes. However, the converse was not true; for there were peptides present in the material from reticulocytes that were not found in the haemoglobin-like material from oocytes.

In the present paper, the substance synthesized under the direction of 9 s RNA is identified as rabbit globin using more sensitive analytical criteria, namely cation-exchange chromatography, paper chromatography and paper electrophoresis of histidine-labelled tryptic peptides. The results show that there is perfect coincidence between histidine-labelled tryptic peptides obtained from reticulocyte β-globin chains and peptides from oocyte-derived β-chains, and that there is an almost perfect coincidence between peptides from reticulocyte α-chains and peptides from oocyte-derived α-chains. Therefore the material made in the oocyte can now be identified as being very similar to normal rabbit globin chains.

Twenty frog oocytes were injected, individually, with approximately 50 nl. of a solution of 9 s reticulocyte RNA (700 μg/ml.) made up in a haemin-containing buffer.

† Paper I in this series is Lane, Marbaix & Gurdon (1971).

The oocytes were incubated at 19°C for 15 hours in a solution containing [2,5-^{3}H] histidine (1 mCi/ml. at 52 Ci/m-mole); the oocytes were frozen at −70°C and were subsequently homogenized in a pH 8·9 buffer containing unlabelled histidine (5 mM) and commercial rabbit haemoglobin (5 mg/ml.). The resulting homogenate was spun at 75,000 ***g*** for 20 minutes and the clear supernatant so obtained was applied to a 140 cm × 1 cm column of G100 Sephadex. The tubes containing the marker rabbit haemoglobin were pooled and the resulting solution of radioactive protein was subjected to cold acid/acetone treatment so as to prepare globin. The α- and β-globin chains were then separated by chromatography on carboxymethyl cellulose, using a pyridine/formic acid gradient (Dintzis, 1961). The preparation of globin chains from 9 s RNA injected oocytes follows exactly the method described in detail, by Lane *et al.* (1971).

The presence of 9 s RNA in the injection mixture is associated with the formation of radioactive material that co-chromatographs with marker rabbit globin chains. Thus the labelled polypeptides from the oocyte-derived material closely resemble the α- and β-globin chains found in normal rabbit reticulocyte haemoglobin. However, oocyte-derived globin prepared in the manner described may contain a 20-fold or greater excess of β- as opposed to α-globin chains. The observed paucity of α-chains may explain the spectrum of peptides obtained in the previous work (Lane *et al.*, 1971). The absence of complete homology between peptides from oocyte-derived material and peptides from rabbit reticulocyte haemoglobin may well have been the direct outcome of having analysed a sample of whole haemoglobin that lacked significant amounts of labelled α-chains. Thus low yield histidine-containing peptides of the α-chain may not have been represented in the tryptic digest of the oocyte synthesized haemoglobin. The fact that normal $\alpha_2\beta_2$ haemoglobin molecules with unequal amounts of labelled α- and β-chains may be obtained is possibly the result of homogenizing with unlabelled marker rabbit haemoglobin under conditions which permit sub-unit exchange (Lane, 1971). The analytical procedures used do not seem to cause a preferential loss of marker α-chains. Moreover the front peak obtained from gel filtration of the whole supernatant does not contain significant amounts of labelled globin chains: one can, therefore, argue that there is probably less α-chain than β-chain present in oocytes injected with 9 s RNA. The underlying cause for this difference is not known. However, one must remember that regulatory mechanisms which operate in the reticulocyte may not do so in the oocyte; moreover the 9 s RNA preparation used may have contained unequal amounts of the α- and β-globin chain messengers.

The [^{3}H]histidine-labelled polypeptides from the α- and β-chain regions of the carboxymethyl-cellulose elution profile were pooled separately and were then mixed with the appropriate marker rabbit reticulocyte α- or β-globin chains uniformly labelled with [^{14}C]histidine. The resulting solutions were lyophilized and the globin chains were then digested with trypsin, as described previously (Lane *et al.*, 1971). The tryptic peptides were then analyzed on a cation-exchange resin as described by Lane *et al.* (1971). Figure 1 shows the elution profile of histidine-containing tryptic peptides from the mixed α-globin chains. The distribution of ^{3}H-labelled peptides coincides perfectly with that of the ^{14}C-labelled marker peptides, except for one supplementary tritium-containing peak; the nature of this additional peak is not as yet known. However, it is possible that this extra peak could be the peptide α-T6b, described by von Ehrenstein (1966). This peptide has phenylalanine-threonine in positions 48–49, and is only present in animals that contain the gene for this α-chain variant: and in the

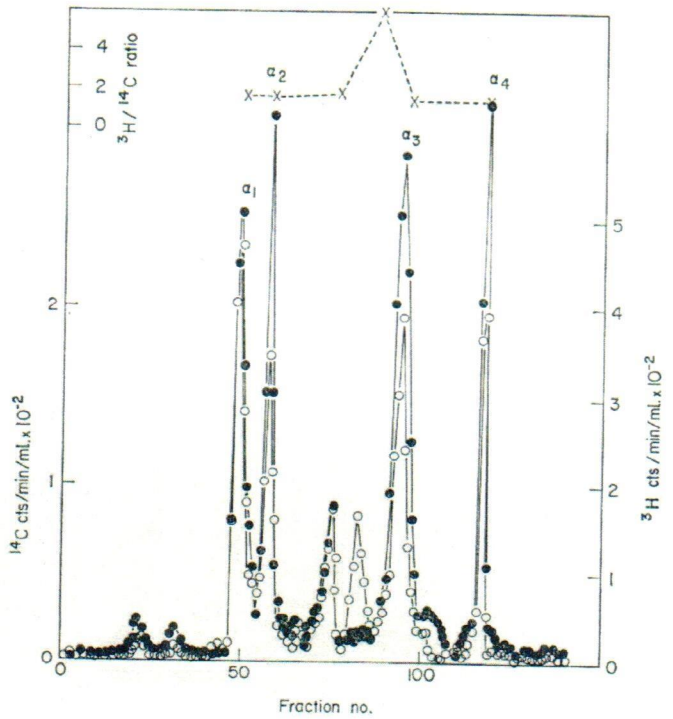

FIG. 1. 20 oocytes were injected, individually, with a solution of rabbit reticulocyte 9 s RNA (at 700 μg/ml.). The oocytes were incubated at 19°C for 15 hr in a medium containing [2,5-^{3}H] histidine (52 Ci/m-mole) at 1 mCi/ml. The procedures for handling oocytes were as described by Lane *et al.* (1971). After incubation, the oocytes were homogenized in 0·7 ml. of a pH 8·9 buffer containing 0·0522 M-Tris, 0·0522 M-glycine, 0·005 M-histidine and commercial rabbit haemoglobin (5 mg/ml.); the homogenate was spun for 20 min at 75,000 ***g*** and the clear supernatant so obtained was applied to a 140 cm × 1 cm column of G100 Sephadex equilibrated in 0·0522 M-glycine/0·0522 M-Tris pH 8·9. The tubes containing the marker rabbit haemoglobin were pooled, and 50 mg of fresh rabbit haemoglobin (obtained as a stroma-free rabbit reticulocyte lysate) were mixed into the solution: after adding a few drops of β-mercaptoethanol the mixture was added dropwise to cold acetone (−20°C) containing 0·1% w/v β-mercaptoethanol and 1·2% w/v 12 N-HCl. The globin so obtained was collected by centrifugation and was chromatographed on a 50 cm × 1 cm column of Whatman CM52 cellulose eluted using a linear (500 ml. × 500 ml.) gradient of 0·2 N-formic acid/0·02 N-pyridine to 1·55 N-formic acid/0·155 N-pyridine. 6-ml. fractions were collected. 100-μl. portions were taken for liquid scintillation counting. The fractions containing the ^{3}H-labelled oocyte derived α-chains were pooled and lyophilized; rabbit reticulocyte α-chains, uniformly labelled with [^{14}C]histidine were then added, and the mixture was digested with trypsin as described by Lane *et al.* (1971). The peptide mixture was analysed on a 75 cm × 0·6 cm column of Technicon type P chromobead cation-exchange resin eluted with an acetic acid/pyridine gradient (Lane *et al.*, 1971). 2-ml. fractions were collected, and 1-ml. portions were taken for counting, suitable corrections being made for quenching. —○——○—, ^{3}H cts/min/ml.; —●——●—, ^{14}C cts/min/ml.; ––×----×––, the ^{3}H/^{14}C ratio, which was determined for each discrete peak of labelled material. The numbered peaks denote those peptides subjected to further analysis by chromatography and electrophoresis.

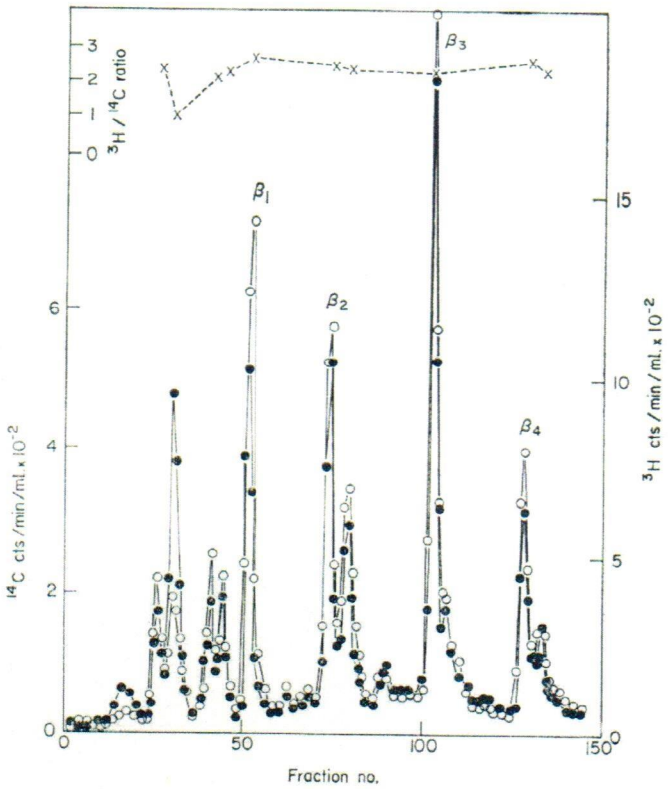

FIG. 2. Rabbit reticulocyte β-chains, labelled with [^{14}C]histidine were mixed with [^{3}H]histidine-labelled oocyte-derived β-chains (derived from the carboxymethyl cellulose eluate) and the mixture was digested, chromatographed and counted as described in the legend to Fig. 1. —○——○—, ^{3}H cts/min/ml.; —●——●—, ^{14}C cts/min/ml.; --×----×--, ^{3}H/^{14}C ratio, which was determined for each discrete peak of labelled material. The numbered peaks denote those peptides subjected to further analysis by chromatography and electrophoresis.

above experiment different rabbits were used to prepare the messenger RNA and [^{14}C]histidine labelled marker globin chains. Figure 2 shows the elution profile of the histidine-containing tryptic peptides from the β-globin chain. The distribution of ^{3}H-labelled peptides coincides perfectly with that of the ^{14}C-labelled marker peptides. However, as can be seen from the low value of the ratio of ^{3}H to ^{14}C radioactivity, there is disproportionately little oocyte-derived material in the peptide material which forms the second peak of the elution profile. This could be the result of injecting a sample of 9 s RNA that happened to be a mixture of two different β-chain messengers, one coding for β^{52} His and the other for β^{52} Asn.

In an attempt to test still further the homology between rabbit reticulocyte globin chains and globin chains synthesized in 9 s RNA injected oocytes, fractions from the column eluate were pooled and the resulting peptides subjected to paper chromatography and paper electrophoresis. Four peptides from the α-chain and four peptides from the β-chain were each analysed by paper electrophoresis at pH 6·5, and by paper chromatography in butanol/acetic acid/pyridine/water mixture. Although

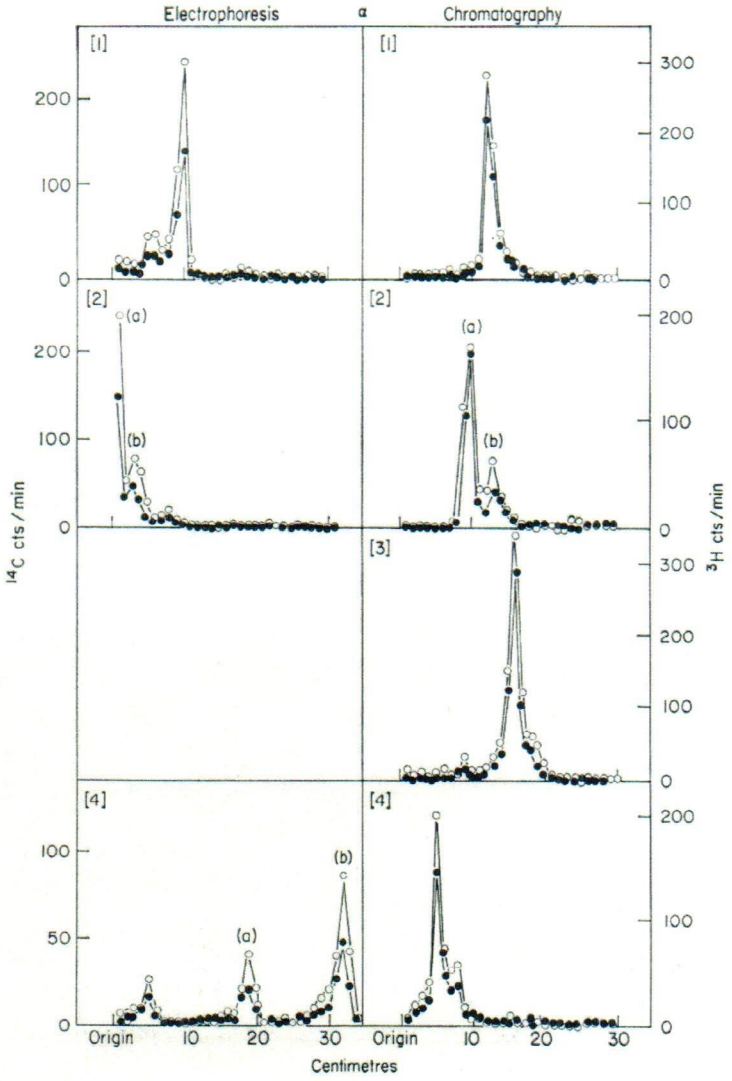

FIG. 3. The fractions corresponding to the peptides marked α_1, α_2, α_3 and α_4 in Fig. 1 were pooled (separately) and the resulting solutions lyophilized. The material in each sample was then divided into 2 portions. One portion was used for paper electrophoresis on Whatman no. 1 paper using a pH 6·5 buffer of pyridine/acetic acid/water (25:1:225, by vol.). Papers were run for approximately 1 hr at 3 kV. The other portion was used for chromatography on Whatman no. 1 paper; papers were run overnight in butanol/acetic acid/water/pyridine (30:6:24:20, by vol.). In both instances the paper was then dried and cut up into 1-cm strips which were added to vials containing 1 ml. of 0·1 N-NH_3. After shaking for 1 hr at room temperature, 12 ml. of an ethoxy-ethanol based scintillant containing 25% Triton X100 were added to each vial. Tests have shown that at least 90% of the radioactivity is eluted using this method. Suitable corrections were made for quenching. —○——○—, ^{3}H cts/min/ml.; —●——●—, ^{14}C cts/min/ml. The numbers in single brackets [4] enable one to deduce the origin of the material used, by comparison with the numbered peaks of Fig. 1. The letters (a) and (b) refer, respectively, to the slower and faster moving peaks of samples that split into two major peaks. The ^{3}H/^{14}C ratios of the peaks are shown in Table 1.

the material analysed was in some instances neither chromatographically nor electrophoretically homogeneous, Figures 3 and 4 show that the distribution of ^{3}H-labelled peptides coincides exactly with that of the ^{14}C-labelled marker peptides. As shown in Table 1, the relative amounts of ^{3}H- and ^{14}C-labelled peptides are not drastically altered by these analytical procedures: moreover, for different peptides (from a given polypeptide chain) subjected to the same analytical and counting procedures, the $^{3}H/^{14}C$ ratios are quite similar.

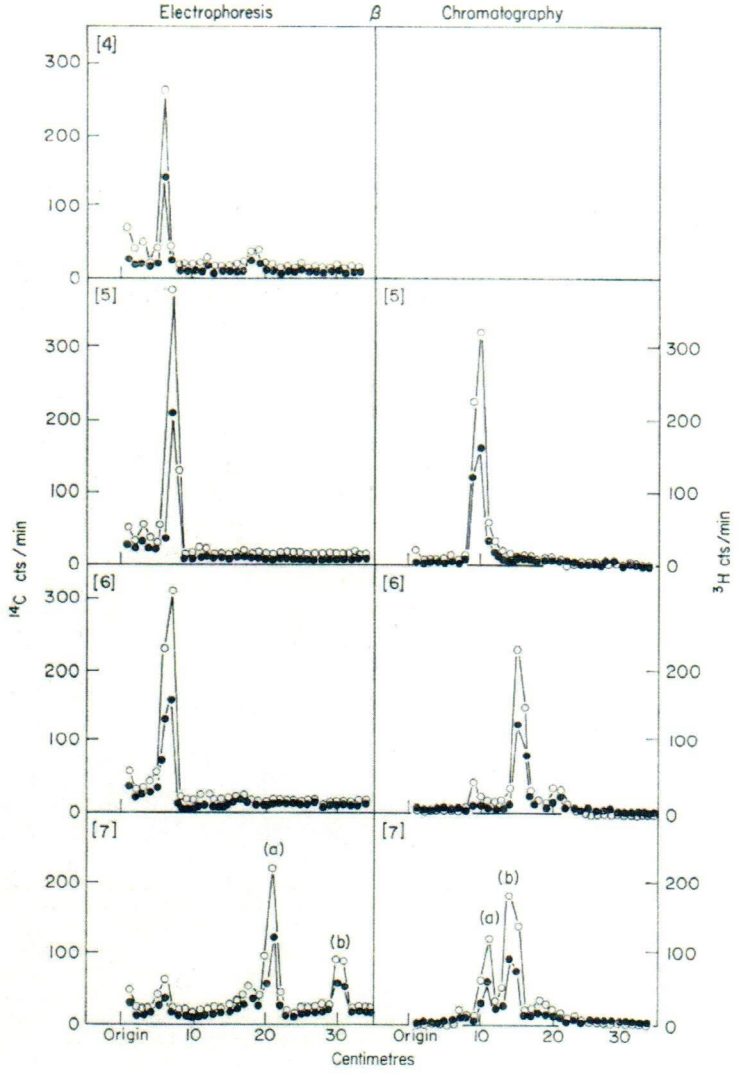

FIG. 4. The fractions corresponding to each of the peptides marked β_1, β_2, β_3 and β_4 in Fig. 2 were pooled separately and the resulting solutions were analysed by the procedures described in Fig. 3. —○——○—, ^{3}H cts/min/ml.; —●——●—, ^{14}C cts/min/ml. The numbers in single brackets [4] enable one to deduce the origin of the material used, by comparison with the numbered peaks of Fig. 2. The letters (a) and (b) refer to the slower and faster moving peaks respectively of samples that split into two major peaks. The $^{3}H/^{14}C$ ratios of the peaks are shown in Table 1.

TABLE 1

The ratio of ^3H (oocyte-derived) to ^{14}C (reticulocyte-derived) radioactivity of histidine-labelled peptides, from α and β chains, after purification by cation-exchange chromatography, paper electrophoresis and paper chromatography

Peptide material analysed	Ratio of $^3H/^{14}C$ radioactivity† Ion exchange chromatography	Paper‡ electrophoresis	Paper‡ chromatography
α_1¶	1·8	1·7	1·2
α_2	1·3	(a) 1·6	(a) 1·0
		(b) 1·8	(b) 1·4
α_3	1·2	—	1·3
α_4	1·4	(a) 1·6	1·6
		(b) 1·8	
β_1¶	2·7	1·9§	—
β_2	2·5	1·8	2·1
β_3	2·3	1·9	1·9§
β_4	2·6	(a) 1·7	(a) 2·0
		(b) 1·6	(b) 2·0

† Counting conditions were similar but not identical in each of the 3 analytical procedures. Therefore one would not necessarily expect the $^3H/^{14}C$ ratio to be the same in each case.

‡ The letters (a) and (b) refer to the slower or faster moving peaks respectively of samples that split into two peaks. See Figs 3 and 4.

§ Value for major peak.

¶ Since less ^{14}C-labelled globin chain was mixed with the oocyte-derived β chain, the absolute value of the $^3H/^{14}C$ ratio is expected to be greater for β chain peptides than for α chain peptides.

These experiments therefore show that 9 s RNA from rabbit reticulocytes, when injected into frog oocytes, causes the synthesis of material that is very similar, if not identical, to rabbit reticulocyte α- and β-globin chains. The oocyte-derived material may not have exactly the same amino-acid sequence as genuine α- and β-chains; the analytical techniques employed may have failed to reveal certain amino-acid substitutions and moreover only the histidine-labelled tryptic peptides have been analysed. Histidine would be expected to label at least 12 and possibly 16 out of the 31 or more peptides which can be obtained, in theory, by tryptic digestion of rabbit globin (von Ehrenstein, 1966; Galizzi, 1971; Braunitzer, Best, Flamm & Schrank, 1966). Nonetheless the methods used so far have failed to reveal any profound difference between oocyte-derived and rabbit reticulocyte globin chains. The extra peptide found in oocyte-derived α-chain material does not provide direct evidence of a translational error: the difference could have arisen subsequent to translation or it could be the result of genetic differences between the rabbits used to prepare messenger RNA and those used to make marker globin chains, and the extra peak may even have resulted from newly synthesized frog proteins that were carried through the purification procedure.

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