EVIDENCE FOR THE SYNTHESIS OF A PRECURSOR OF CARP PROINSULIN IN A CELL FREE TRANSLATION SYSTEM

Tom A. RAPOPORT*, Wolfgang E. HÖHNE**, Dietmar KLATT**, Siegfried PREHN** and Volkmar HAHN*

Zentralinstitut für Molekularbiologie der Akademie der Wissenschaften, Berlin-Buch, DDR and
**Institut für physiologische und biologische Chemie der Humboldt-Universität zu Berlin, DDR*

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1. Introduction

Until recently the cell free translation of proinsulin mRNA* has not been successful. This is due to the difficulties in obtaining sufficient amounts of endocrine tissue of the pancreas and to the high content of ribonuclease. The islets of Langerhans of fish (Brockmann bodies) are very suitable objects. They can be isolated with only little contamination by exocrine tissue and are much bigger than those of mammals.

In previous experiments we characterized the islets of carp (*Cyprinus carpio*) by their ability to synthesize proinsulin in tissue culture [1] and by their content of insulin (unpublished results). The synthesis of a proinsulin-like material could also be shown using polysomes of the carp islets in a rat liver cell free system [2].

While the work was in progress two relevant papers appeared. Yip et al. reported the translation of total RNA from rat islets and tumor tissue in the cellular system of oocytes [3]. Lomedico and Saunders used a wheat germ cell free system for the translation of poly A^+-RNA from adult dog or fetal bovine pancreas [4]. Whereas in both cases products larger than proinsulin appeared to be synthesized there was a discrepancy with respect to the molecular weights (18 000 and 12–13 000 dalton, respectively).

The present paper deals with the cell free translation in a wheat germ system of poly A^+-RNA from Brockmann bodies of the carp. It is shown that an immunoreactive insulin-polypeptide is synthesized which may be a precursor of proinsulin.

* *Abbreviations:* mRNA, messenger RNA; poly A^+-RNA, RNA containing the 3'-terminal poly A sequence; SDS, sodium dodecylsulfate.

2. Materials and methods

Islets of Langerhans of the carp (Brockmann bodies) were obtained as previously described [1]. They were collected at −20°C in a glycerol buffer (50% glycerol; 10 mM Tris/HCl, pH 7.4; 10 mM NaCl; 1.5 mM $MgCl_2$) containing heparin (250 U/ml), bentonite (0.5%; sometimes omitted) and Contrycal as a protease inhibitor (10 000 U/ml). The homogenisation of the tissue was carried out essentially according to Nokin et al. [5]. The islets were transferred into a mortar with liquid nitrogen and thoroughly ground to a fine powder. When the nitrogen had evaporated the pink powder was immediately transferred into a mixture of equal volumes of Tris-buffer (0.1 M Tris/HCl, pH 9.0; 0.1 M NaCl; 1 mM EDTA; 1% SDS) and phenol (buffer saturated) : chloroform : isoamylalcohol (50 : 50 : 1). For the islets of 20 carp 20 ml of the whole mixture were used. The tissue was immediately homogenized for 40 s in a Virtis homogenizer at position 20. Thereafter the mixture was shaken for 15 min at 4°C. Following centrifugation (5000 *g* for 10 min) the aqueous phase was extracted three additional times at 4°C with phenol–chloroform and then twice with chloroform. The RNA was precipitated with 1/10 volume 1 M sodium acetate and 2.5 volumes ethanol at −20°C. Preparation of poly A^+-RNA was performed by poly U-Sepharose chromatography [6]. The

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elution of the bound RNA was carried out with 0.2% SDS at 50°C. The RNA was precipitated with 1/10 volume 5 M sodium acetate, 2 volumes ethanol and 1 volume diethylether at −20°C. It was collected by centrifugation (5000 *g* for 30 min), dissolved in bidistilled water and stored at −20°C. For all steps in the preparation of the RNA sterilized glass ware, autoclaved buffer solutions and freshly distilled organic solvents were employed. Siliconized glass ware was used starting with the elution of the poly A^+-RNA through all subsequent steps.

Sucrose gradient centrifugation of RNA was performed in 5–20% linear sucrose gradients in 10 mM Tris/HCl, pH 7.4; 10 mM NaCl; 10 mM EDTA; 0.2% SDS; 0.1% Na-deoxycholate at 25 000 rpm at 15°C for 15 h in a Beckmann SW 40 rotor.

Cell free translation was carried out with the wheat germ system [7]. The wheat germ extract (S-23) was prepared freshly.

For product analysis the cell free translation was performed in 250 μl assays which yielded about 2.5×10^5 cpm with 5 μg poly A^+-RNA from carp islets. After 1 h of incubation 2 mg bovine insulin was added and the mixture precipitated with TCA (final concentration 5%). After three washes with TCA the precipitate was extracted twice with 1 ml 5 M acetic acid/0.15 M NaCl and the extract applied to a Sephadex G-75 column (1.5 × 100 cm) equilibrated with this solvent. Aliquots of the eluted fractions were dried, dissolved in a small volume of water and the radioactivity counted in a dioxan-based scintillation cocktail. The fractions containing the proinsulin-like material were pooled and dried in a rotary evaporator. The material was dissolved in 1 ml 0.1 M $(NH_4)_2CO_3$.

Antiserum against carp insulin coupled to Sepharose 4-B (200 mg) was suspended in 440 μl IRI-buffer (0.04 M phosphate buffer pH 7.4; 0.3% human serum albumin; 0.1 M NaCl; 5 mM NaN_3) and 60 μl of the proinsulin-like material were added. The incubation was carried out at 4°C for 12-15 h under continuous shaking. 2 ml cold 0.9% NaCl were added and, after centrifugation, the precipitate washed twice with 0.9% NaCl. The precipitate and the dried supernatant were dissolved in 0.2 ml Hyamine (60°C for 5 h) and counted in a dioxan-based scintillation cocktail. The binding capacity of the matrix-bound antibody was determined with ^{125}J-carp insulin. 200 mg matrix-bound antibody bind approximately 160 ng carp insulin.

Antibodies to carp insulin were produced in guinea pigs [8]. They were coupled to BrCN-activated Sepharose 4-B according to standard procedures.

The carp insulin used for the immunisation was prepared in milligram amounts from islets of 500 carp. It was purified by gel filtration, ion exchange chromatography and by insulin-specific precipitation with Zn^{2+}-ethanol. The crystalline material displayed two bands in the disc-electrophoresis. However, according to chemical properties, biological tests, molecular weight and amino acid composition both components are insulin like (unpublished results).

SDS-electrophoresis with the proinsulin-like material was carried out as follows. The material was desalted by passage through a Sephadex G-25 column (1.5 × 10 cm) which was equilibrated with 0.1 M $(NH_4)_2CO_3$. The fractions of the void volume were dried in a rotary evaporator and the $(NH_4)_2CO_3$ removed in vacuum. The material was finally dissolved in electrophoresis buffer (10 mM phosphate buffer pH 7.0; 1% SDS; 2% 2-mercaptoethanol; 6 M urea) and bovine proinsulin and chymotrypsinogen were added as internal markers. The sample was heated to 100°C for 3 min. The electrophoresis was performed in 15% slab gels according to Weber and Osborn [9] except that 6 M urea was present in the gels. The positions of the marker proteins (ovalbumin, chymotrypsinogen, myoglobin, cytochrome *c*, proinsulin, insulin) were identified by UV-scanning (Gilford photometer). The gel was cut in discs of 1 mm thickness, dissolved in 0.5 ml Protosol/water (9 : 1, v/v) at 50°C over night and counted in a toluol based scintillation cocktail.

3. Results and discussion

15 mg total RNA could be obtained from the islets of about 100 carp (300–600 g body weight). The preparation was largely undegraded as judged from the 28 S : 18 S ratio in the sucrose gradient (fig.1). With the method used the poly A tract of the poly A^+-RNA remains intact [10] and little DNA and nuclear RNA is extracted at 4°C [11].

Fig.2 shows the translation of the poly A^+-RNA in a wheat germ cell free system as a function of time

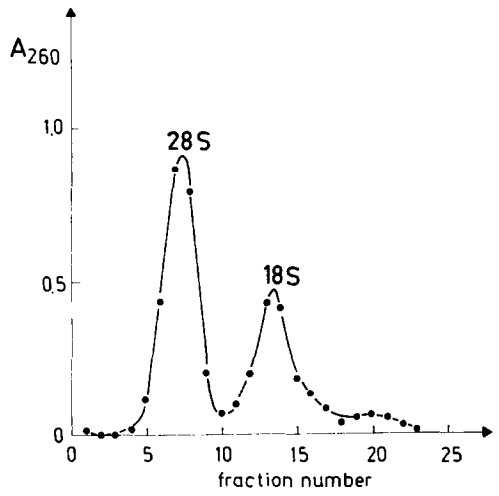

Fig.1. Sucrose gradient centrifugation of total RNA from carp islets. From the areas under the RNA-peaks a ratio of 28 S : 18 S of 2.3 was calculated which indicates that the ribosomal RNA is undegraded.

and the amount of RNA in the assay. For comparison the respective curves for globin mRNA are given. Both RNAs are equally efficient on a weight basis.

For the product analysis of the cell free translation the TCA-precipitate was extracted with acetic acid to dissolve insulin-like material. About ¼–½ of the incorporated radioactivity was solubilized. Carrier bovine insulin was added. It does not crossreact with the anti-carp insulin antibodies even in milligram amounts (unpublished results). Fig.3 shows the chromatogram of the extract on Sephadex G-75. A peak at the position of proinsulin is noted.

The insulin-like nature of the material was proved by immunological means. The material from the peak fractions of the Sephadex column was pooled. After removal of the acetic acid it was incubated with a great excess of anti-carp insulin antiserum coupled to BrCN-activated Sepharose 4-B. Table 1 shows that 90% of the radioactivity is bound to the matrix coupled antiserum. Essential controls for nonspecific binding

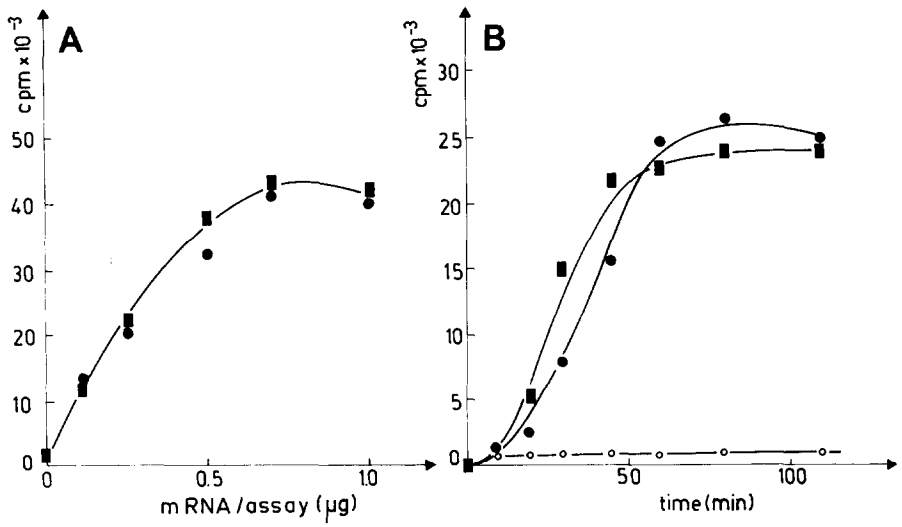

Fig.2. Translation of poly A⁺-RNA from carp islets and of globin mRNA in a wheat germ cell free system. The assays contained in final concentrations: 4.0 mM Mg^{2+} (determined by atom absorption); 45 mM K^+ (determined by flame photometry); 20 µM amino acids (chromatographically pure) except leucine; 2 mM dithioerythritol; 16 mM tris/HCl, pH 7.6; 0.2 mM GTP; 1 mM ATP; 8 mM phosphoenolpyruvate; 40 µg/ml pyruvate kinase (the latter four substances were tested enzymatically); 5 µCi ^{3}H-leucine (Amersham, 50-60 Ci/mmol); 20 µl S-23 from wheat germs and 1 µg mRNA (assuming 1 mg/ml ≅ A_{260} = 25) (the latter additions per 50 µl assay). The incorporation at 25°C into hot TCA-precipitable material was determined by the filter technique (A) Dependence on the amount of mRNA. The points represent the incorporated radioactivity in a 50 µl assay. ▪ poly A⁺-RNA, • globin mRNA. (B) Time dependence. The points represent the incorporated radioactivity in a 30 µl assay. ▪ poly A⁺-RNA, • globin mRNA.

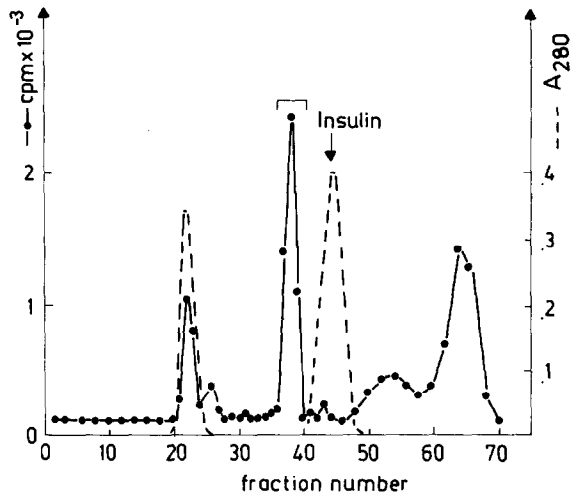

Fig.3. Gel filtration of the poly A^+-RNA directed translation products soluble in acetic acid. The TCA-precipitate of the products of the cell free translation was extracted with 5 M acetic acid/0.15 M NaCl and applied to a Sephadex G-75 column. Fractions of 2.0 ml were collected and 1.0 ml used for counting of the radioactivity. Bovine insulin was added as a carrier. The indicated fractions which contain the pro-insulin-like material were pooled and used for the subsequent characterisation.

include the incubation with Sepharose-coupled control serum of non-immunized guinea pigs and the preincubation of the antibodies with excess of unlabelled carp insulin. The controls show that the binding is specific and that the material is insulin-like.

Since gel filtration on Sephadex G-75 gives only a rough estimate of the molecular weight SDS-electrophoresis was performed with the immunoreactive polypeptide. Fig.4 shows that it has a molecular weight of 12–14 000 dalton and that it is distinctly larger than proinsulin.

This result is in agreement with that of Lomedico and Saunders [4] and in contrast to that of Yip et al. [3]. The possibility remains that in his system oocytes produced preproinsulin of a molecular weight of 18 000 dalton whereas in the cell free systems from wheat germs a protease converts it to a smaller peptide. However, the finding of a homogeneous peak of 12–14 000 dalton in gel filtration and SDS-electrophoresis would require a specific cleavage which would be surprising to find in wheat germs. Furthermore, a high level of unspecific binding of

Table 1

Immunoreactivity of the proinsulin-like material. The proinsulin-like material of the fractions indicated in fig.3 was pooled and incubated with the following additives for 12–15 h. The preincubation with unlabelled carp insulin was carried out with 10 μg for 12 h at 4°C

Additive	Radioactivity bound to Sepharose 4-B (cpm)
Total counts	468 ± 25
Anti carp insulin antiserum coupled to Sepharose 4-B	419 ± 14
Serum of non-immunized guinea pigs coupled to Sepharose 4-B	61 ± 3
Anti carp insulin antiserum coupled to Sepharose 4-B and preincubated with excess of unlabelled carp insulin	67 ± 10

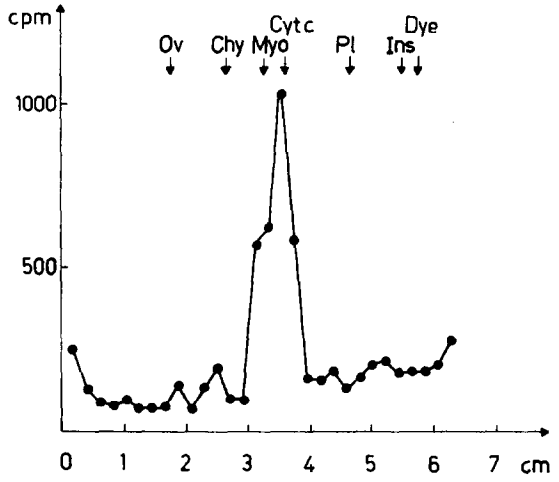

Fig.4. Determination of the molecular weight of the pro-insulin-like material by SDS-gel electrophoresis. Bovine pro-insulin (Pro, 9000 dalton) and chymotrypsinogen A (Chy, 25 000 dalton) were added as internal markers to the pooled fractions of the gel filtration in fig.3. The other marker proteins, ovalbumin (Ov, 45 000 dalton), myoglobin (Myo, 17 800 dalton), cytochrome *c* (Cytc, 12 400 dalton) and insulin (Ins, 6000 dalton) were run on separate slots of the gel. The molecular weight of the radioactive material was estimated from a semilogarithmic plot to be 12–14 000 dalton.

radioactive material to the insulin antibodies makes the finding of Yip et al. unconvincing.

The present study is in accord with the cell free synthesis of a proinsulin-like material by polysomes of carp islets which were released from the membranes [2]. Although the product appeared to have the same molecular weight as proinsulin in gel filtration it could not be converted to insulin by incubation with trypsin. This property is also observed with the product of the cell free translation in wheat germs (unpublished results).

The occurrence of a preproinsulin of 12–14 000 dalton as compared to 9000 dalton for proinsulin fits with the hypothesis of Blobel and Sabatini [12] that proteins synthesized on membrane bound polysomes and destined for the export from the cell need an extra piece (*N*-terminal) for the attachment of the nascent chains to the endoplasmic reticulum.

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References

[1] Lukowsky, A., Prehn, S. and Rapoport, T. A. (1974) Biochim. Biophys. Acta 359, 248–252.
[2] Rapoport, T. A., Prehn, S., Lukowsky, A. and Junghahn, I. (1974) Acta Biol. Med. German 33, 953–961.
[3] Yip, C. C., Hew, C.-L. and Hsu, H. (1975) Proc. Natl. Acad. Sci. USA 72, 4777–4779.
[4] Lomedico, P. T. and Saunders, G. F. (1976) Nucleic Acids Res. 3, 381–391.
[5] Nokin, P., Burny, A., Cleuter, Y., Huez, G., Marbaix, G. and Chantrenne, H. (1975) Eur. J. Biochem. 53, 83–90.
[6] Molloy, G. R., Jelinek, W., Saldiff, M. and Darnell, J. E. (1974) Cell 1, 43–53.
[7] Roberts, B. E. and Paterson, B. M. (1973) Proc. Natl. Acad. Sci. USA 70, 2330–2337.
[8] Ziegler, M., Karg, U. and Lippmann, H. G. (1969) Wiss. Zeitschr. Karl-Marx-Univ. Leipzig, Math.-Naturwiss. R., 18. Jg. H. 4, 609–615.
[9] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[10] Brawerman, G. (1975) Cell 5, 271–280.
[11] Georgiev, G. P., Ryskov, A. P., Coutelle, Ch., Mantieva, V. L. and Avakyan, E. R. (1972) Biochim. Biophys. Acta 259, 259–283.
[12] Blobel, G. and Sabatini, D. (1971) in: Biomembranes (Manson, L. A., ed) Vol. 2, 193–195, Plenum Publishing Corp., New York.