Quantitation of chordin in developing *Huso huso* embryos and larvae by radioimmunoassay

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Chordin is a protein discovered in the notochord cells of the representatives of Acipenseridae; giant sturgeon, stellate sturgeon and sterlet. Some characteristics of the purified chordin preparation which justify its use in radioimmunoassay are described. A sensitive competitive-binding double-antibody radioimmunoassay for chordin is described by which its content in the extracts from giant sturgeon embryos and larvae has been measured. It is shown that chordin biosynthesis started in the embryos from stage 32.

Chordin *Tissue-specific protein* *Radioimmunoassay* *Type II collagen* *Embryonic differentiation marker*

1. INTRODUCTION

Chordin is a protein antigen discovered in giant sturgeon (*Huso huso*), stellate sturgeon (*Acipenser stellatus*) and sterlet (*A. ruthenus*) notochord core cells [1,2]. It was shown to be a notochord-specific but species non-specific antigen within the Acipenseridae [1]. Chordin has been purified from other antigens of the giant sturgeon notochord core cell extract [1,3]. The antigenic determinants of chordin are stable to heat inactivation (56°C, 2 h), to the action of DNase and RNase and sensitive to protease digestion (papain or trypsin) [1]. Amino acid analysis of the chordin preparation has shown the prevalence of acidic amino acids. The isoelectric point of chordin is near 1. The apparent M_r of chordin as determined on a calibrated column of AcA-34 Biogel is 100000 [3].

Here, a competitive-binding double-antibody radioimmunoassay with a sensitivity less than 0.1 ng/100 μl was used for the quantitation of chordin in the extracts from giant sturgeon embryos and larvae. It was established that chordin biosynthesis started from stage 32 [4], much later than the biosynthesis of type II collagen of which the content was measured earlier in the same embryos.

2. MATERIALS AND METHODS

2.1. *Biological materials*

Giant sturgeon embryos and larvae were obtained from the Volgograd sturgeon fish hatchery. Determination of embryonic development stages was performed according to the tables of Dettlaff and Ginsburg [4]. Only embryos of identical stages were chosen as samples for experiments. Jelly coats were removed and the embryos were frozen and stored in liquid nitrogen until use.

2.2. *Preparation of extracts from embryos*

The thawed material (20 embryos) was adjusted with T buffer (0.1 M NaCl, 0.5% Triton X-100, 0.02% NaN_3, 0.01 M Na-phosphate, pH 7.4) to 2 ml. Homogenation was performed with a glass pestle in a nitrocellulose test-tube for an SW-39 rotor with 50 strokes at 4°C. After centrifugation at 20000 × g for 10 min the soluble extract located between the fat film and the precipitate was removed and used for chordin determination.

2.3. *Radioimmunoassay*

Chordin purification and its labelling in vitro by ^{125}I have been described [3]. Rabbits were immunized by the notochord core cell homogenate [1] and the antiserum obtained was diluted 1:600

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before experiments with T buffer supplied with 0.5% bovine serum albumin. Aliquots of the extract (100 μl) were incubated with rabbit antiserum (40 μl) at 20°C for 3 h. Aliquots of ^{125}I-chordin (about 12000 cpm in 10–20 μl) were added and the mixture was incubated again at 20°C for 3 h. Sheep serum against rabbit γ-globulin was then added for 20 h at 4°C. Statistical treatment of the data was performed as in [5]. The true significance of the determined values lay within the limits shown in figs 3 and 4 with a probability of 95%.

2.4. *Analytical procedures*

SDS-PAGE was performed according to Laemmli [6] in slabs with $T = 15$ and $C = 2.67$. Crossed and line immunoelectrophoresis was carried out as described in [1]. To analyze the immunoprecipitate-derived chordin, line immunoelectrophoresis of ^{125}I-chordin mixed with the notochord core cell extract was first performed, then the corresponding immunoprecipitation line was dissected from the agarose gel and the chordin immune complex was dissociated by the sample buffer.

3. RESULTS

3.1. *Characterization of the purified chordin preparation*

In the previous work purification of chordin from other antigens of the notochord core cell extract was reported. A final preparation was obtained in which no other antigens were detected by crossed immunoelectrophoresis when antiserum against the notochord core homogenate was used [1,3]. Here we present some other characteristics of the chordin preparation labelled with ^{125}I. After SDS-PAGE of the chordin preparation the radioactivity distributes in a broad area (fig.1, lane 1). A similar distribution of the radioactivity of the gel is observed after electrophoresis of the immunoprecipitate-derived chordin (fig.1, lane 2). The distribution of the proteins from the initial notochord core extract after SDS-PAGE as visualized by Coomassie staining is shown in lane 3 of fig.1. The proteins form the usual narrow bands under the same conditions. There are no 'normal' ^{125}I-labelled proteins in the purified chordin preparation (lanes 1,2).

Crossed immunoelectrophoresis of the mixture of ^{125}I-chordin with unlabelled chordin was performed to characterize the labelled chordin preparation (fig.2). One can see that ^{125}I-chordin behaves as an individual antigen. No other precipitation arc was formed in the gel after crossed immunoelectrophoresis in the case when crude notochord core cell extract had been substituted for unlabelled chordin (not shown).

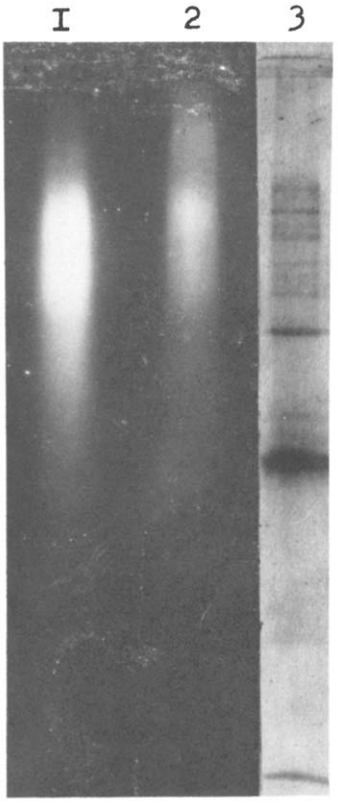

Fig.1. SDS-PAGE. (1) Purified ^{125}I-chordin preparation; (2) the same after line immunoelectrophoresis and dissociation of chordin immune complex; (3) proteins of the notochord core cell extract. (1,2) Autoradiography, (3) staining with Coomassie blue R-250.

3.2. *Radioimmunoassay of chordin in the extract from embryos and larvae*

The calibration curve for chordin determination is presented in fig.3. Less than 0.1 ng chordin can be detected in this double-antibody system. The measurements of chordin concentrations in the extracts from giant sturgeon embryos at different stages of development and from some larvae have been performed by radioimmunoassay. As seen

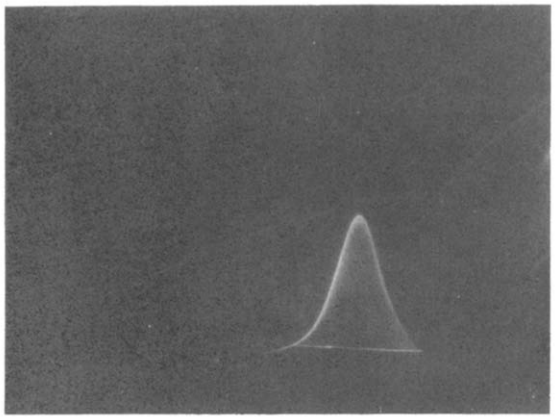

Fig.2. Crossed immunoelectrophoresis of the mixture of ^{125}I-chordin and unlabelled chordin (autoradiography). Antiserum against the notochord core homogenate was used.

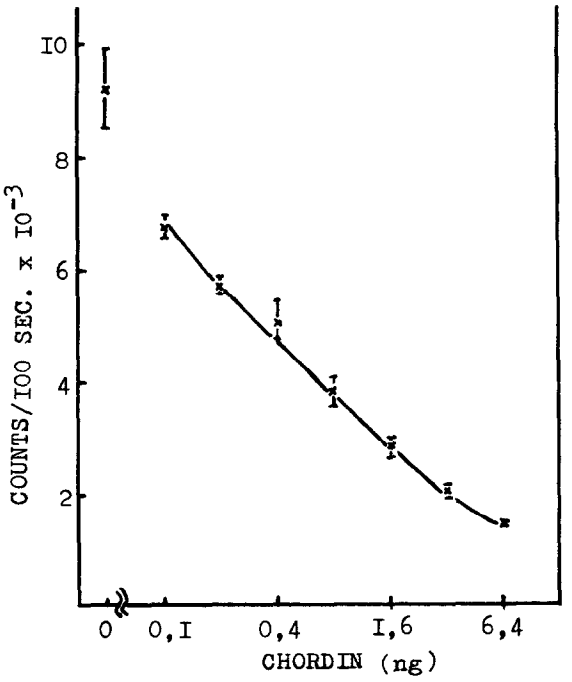

Fig.3. Radioimmunoassay calibration curve for chordin.

from fig.4, curve 1, the appearance of chordin antigenic determinants is first reliably registered at stage 32 and the most prominent increase in the chordin content occurs on the third and fourth days after hatching. Curve 3, which depicts type II collagen content in *H. huso* embryos as determined by radioimmunoassay, is reproduced from [5] for comparison. This collagen, abundant in the notochord sheath [7,8], is detected in the embryos much earlier than chordin.

4. DISCUSSION

When a protein is to be determined by radioimmunoassay the homogeneity of a standard and labelled species is usually estimated by gel electrophoresis and other appropriate methods. In our case the problem could not have been solved in a simple way since the heterogeneity of chordin is likely to be its inherent property. Indeed, chordin displays a broad distribution not only on polyacrylamide gels containing SDS (fig.1), but also on gels containing urea and on agarose gels without denaturing agents (not shown), nor does it behave like a homogeneous protein during chromatography on DEAE-cellulose [3]. On the other hand, chordin behaves as an individual antigen in crossed immunoelectrophoresis ([1,3], see also fig.2). Such properties of chordin may reflect the fact that chordin contains carbohydrate chains of different length. This question was not investigated here. We tried to stain chordin for protein either on nitrocellulose filters or on polyacrylamide gels but failed to do so. 12.6 μg chordin were not stained with amido black on nitrocellulose filters despite the fact that the employed modification [9] enabled us to visualize 0.1 μg bovine serum albumin (the presence of chordin on filters was checked by counting ^{125}I radioactivity). Neither chordin nor impurities have been visualized by Coomassie staining or silver staining [10] after SDS-PAGE.

Several lines of evidence indicating the suitability of our chordin preparation for radioimmunoassay can be listed here. (i) No contaminating antigens were detected in the purified chordin preparation by crossed immunoelectrophoresis when an anti-whole-tissue serum was used [1,3]. (ii) No protein impurities which can be stained with amido black, Coomassie blue or by the silver method have been visualized in the purified chordin preparation. (iii) The preparation contained no contaminants that could be detected on polyacrylamide gels after labelling with ^{125}I. On this basis we conclude that the isolated chordin preparation [3] is suitable for radioimmunoassay

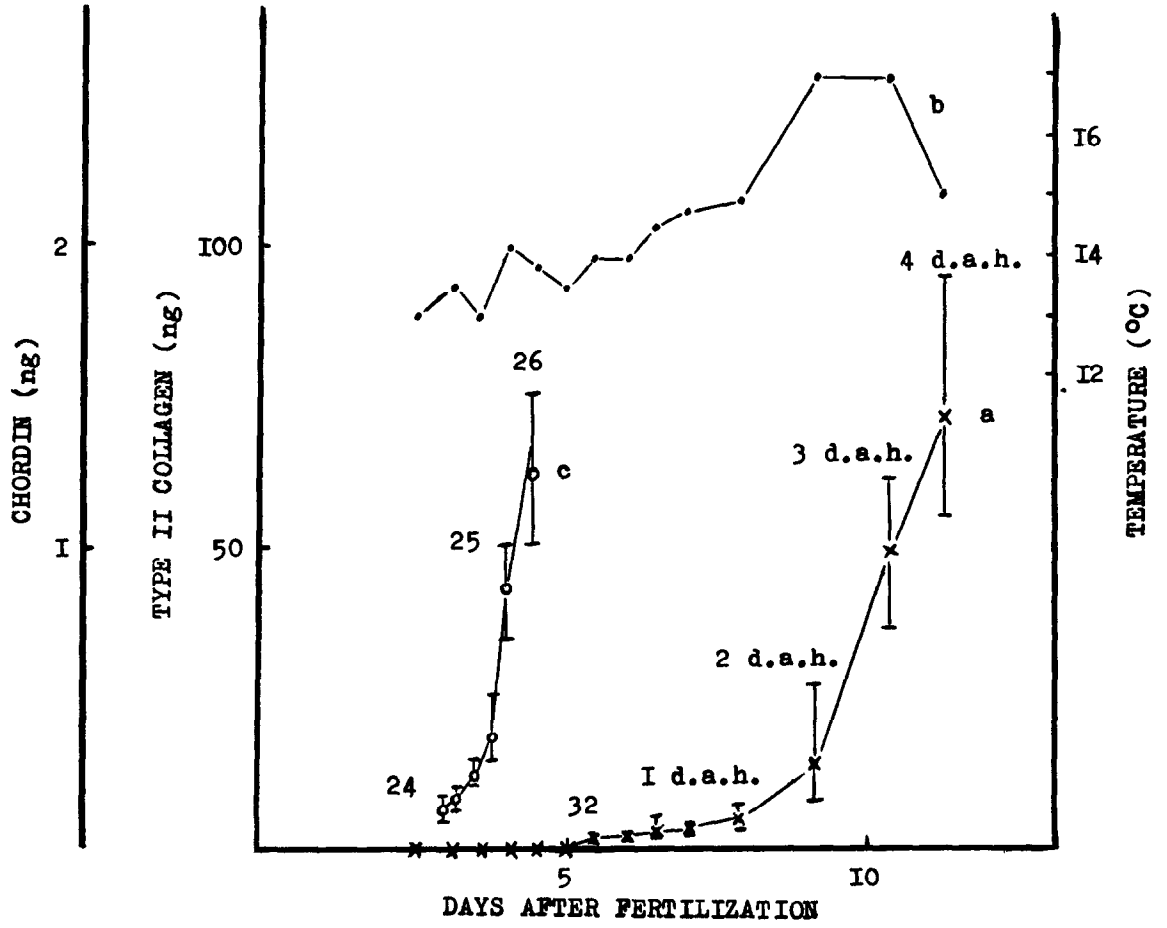

Fig.4. Quantity of chordin per embryo or larva determined by radioimmunoassay. (a) Chordin, (b) temperature (°C) at sampling, (c) quantity of type II collagen per embryo, depicted from [5]. Numbers in the figure show stages of embryonic development; d.a.h., days after hatching.

both as a standard and as a labelled species to detect binding.

As the onset of chordin biosynthesis in giant sturgeon embryos we assume stage 32 ('the tip of the tail touching the head'). At this stage the amount of chordin in the sample differs statistically reliably from the 'zero standard' (a point on the calibration curve where the buffer was substituted for unlabelled chordin). In the previous work we registered the appearance of type II collagen in giant sturgeon embryos during stage 24, i.e. the first stage of organogenesis ([5], see curve 3 in fig.4). The most probable site where type II collagen first appears in the embryo is the notochord sheath [11]. In spite of the fact that the sensitivity of radioimmunoassay for collagen was only 2 ng/100 μl, chordin was registered in the embryos much later. By now 2 distinct biochemical criteria of notochord cell differentiation are available: collagen biosynthesis in the sheath and chordin biosynthesis in the core cells.

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