Localisation and characterization of acid mucopolysaccharides in the early chick blastoderm

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SUMMARY

Acid mucopolysaccharides in the extracellular compartment of early chick blastoderms (16 h of incubation) were labelled with tritiated glucosamine and/or [35S]sulphate. The incorporation pattern was studied autoradiographically. Treatment with testicular hyaluronidase revealed a testicular hyaluronidase-sensitive fraction, mainly at the periphery of Middle Layer and Deep Layer cells, and a testicular hyaluronidase-resistant fraction, mainly at the ventral side of the Upper Layer.

A biochemical analysis, utilizing chondroitinase ABC and nitrous acid, followed by cellulose acetate electrophoresis, demonstrated the synthesis of a non-sulphated fraction, i.e. hyaluronic acid and/or chondroitin, and a sulphated fraction, comprising two undersulphated components, i.e. chondroitin sulphate, and heparan sulphate or heparin. The appearance of different AMPS in specific areas of the early chick blastoderm is regarded as an early specialization of the extracellular compartment.

INTRODUCTION

It is generally accepted that the extracellular matrix plays an important role in cellular interactions, necessary for normal morphogenesis (Bernfield & Wessels, 1970). The presence or the synthesis of acid mucopolysaccharides (AMPS) in the extracellular matrix has been demonstrated in the chick embryo at different stages of development, and their correlation with cellular interactions has been suggested (Franco-Browder, De Rydt & Dorfman, 1963; Kvist & Finnegan, 1970; Manasek, 1970; Manasek et al. 1973; O’Hare, 1973; Abrahamsohn, Lash, Kosher & Minor, 1975; Pratt, Larsen & Johnston, 1975; Solursh, 1976). In this study, the extracellular matrix of the early chick blastoderm, prior to the establishment of recognizable cartilaginous material, was examined for AMPS, using autoradiography and cellulose acetate electrophoresis. We wanted to investigate whether the localization of the labelled products and their nature, could give some insight into the significance of these products.

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MATERIALS AND METHODS

White Leghorn eggs from commercial stock were incubated for 16 h at 38 °C to yield blastoderms of stage 5–6 (Vakaet, 1970), corresponding to Hamburger & Hamilton's stage 3+ (1951). They were explanted according to the procedure of New (1955), and incubated at 38 °C for 30 min. Thereafter the blastoderms were cultured in a medium, containing 0.5 ml egg white and 0.5 ml Ringer solution with 3 mg agar, in the presence of 15 μCi D-[6-3H]glucosamine hydrochloride (19 Ci/mmol) or 70 μCi [35S]sulphate (230 Ci/mmol) (The Radiochemical Centre Ltd., Amersham) for 1 h. Only normally developed embryos were used further.

The blastoderms destined for autoradiography were fixed overnight, at room temperature, in 10% (w/v) aqueous formalin, containing 0.5% (w/v) cetylpyridinium chloride, 2% (w/v) calcium chloride and an excess of calcium carbonate. The tissues were dehydrated in a graded series of alcohol, cleared in xylene, and embedded in paraffin wax. Sections, 8 μm thick, were deparaffinized in xylene, and hydrated. Sections of blastoderms cultured with tritiated glucosamine, were treated with a 0.1% (w/v) solution of ovine testicular hyaluronidase Type II (Sigma Chemical Co.) (365 NF units/mg) in 0.1 M phosphate buffer (pH 5.5) at 38 °C for 5 h. Control incubations were performed on alternate sections in a buffer solution containing the enzyme, inactivated in a boiling-water bath for 10 min. The sections were coated by dipping into Ilford L4 Nuclear Emulsion, diluted 1/1 (v/v) with distilled water, at 45 °C. The slides were exposed for 6 weeks at 4 °C, and processed further according to the method of Caro, Van Tubergen & Kolb (1962). Control incubations without labelled precursors, and incubations with the labelled precursors but cultured at 4 °C, were carried out. A total of 33 blastoderms was studied.

The amount of labelled macromolecules in three groups of three blastoderms and their fixing medium was measured by chromatographing (a) the in vacuo concentrated fixing medium on a PD-2 prepacked column (Pharmacia, Uppsala, Sweden), and (b) the extract of the formerly fixed blastoderms in 4 M guanidin hydrochloride on an analogous column.

The AMPS were further identified using cellulose acetate electrophoresis after culturing the blastoderms with the two precursors in the same medium, and under the same conditions as mentioned above. For each test, five to ten blastoderms were frozen, thawed, homogenized, and digested with papain (Sigma Chemical Co.) according to the method of Antonopoulos, Gardell, Szirmai & De Tyssonsk (1964). The digest was boiled, cooled and centrifuged. The supernatant was dialysed for 2 days against deionized water at 4 °C, and lyophilized. Aliquots of the lyophilizate were treated with either (1) nitrous acid or (2) chondroitinase ABC (Sigma Chemical Co.), respectively according to Dische & Borenfreund (1950), and Yamagata, Saito, Habuchi & Suzuki, (1968). Undegraded AMPS were recovered from the crude extract using dialysis.
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Fig. 1. Outline of a transverse section through the anterior part of the primitive streak of a chick blastoderm at stage 6.

The samples were again lyophilized and resolubilized in a minimal volume. The different AMPS present in the mixture were separated, using cellulose acetate electrophoresis in 0.05 M lithium chloride in 0.01 M HCl (pH 2.1), and in 0.05 M natrium barbital (pH 8.6). Each experiment was performed at least three times. Reference samples of AMPS, i.e. hyaluronic acid, chondroitin sulphate A, B and C (Sigma Chemical Co.), and heparin (Leo Pharmaceutische Producten) were co-electrophoresed. The samples were exposed to a potential gradient of 10 V/cm for 45 min. The cellulose acetate membranes were stained according to Breen, Weinstein, Andersen & Vess (1970). The dried membrane was cut in 2 mm strips and solubilized. The radioactivity in each strip was counted in a liquid scintillation counter (Packard Tri-Carb 3380).

RESULTS

Autoradiography

The incorporation of precursors was examined during the period that active ingression is occurring in the primitive streak. At this stage the regression of Hensen's node has not yet started, and the anlage of the head process is not yet present. The layers of the germ are indicated by Upper Layer (UL) (closest to the vitelline membrane), Deep Layer (DL) (comprising the various generations of the endoblast: endophyll, primary DL or hypoblast and definitive DL or endoblast) and Middle Layer (all cells lying between UL and DL). A schematic representation of a section through the anterior half of the primitive streak is shown in Fig. 1.

After incorporation of tritiated glucosamine for 1 h, all autoradiographs show a typical incorporation pattern of tritium-containing macromolecules (Fig. 2A, B, D). A high density of grains is present at the periphery of ML and DL cells. Moreover, a sharp positivity is observed at the ventral side of the UL, where electron microscope studies (Low, 1967) detect a basal lamina, and at the apical side of the UL in the extraembryonic region. It is remarkable, that during their ingestion, the UL cells acquire the capacity to synthesize these molecules.
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Fig. 3. Autoradiograph (focused on extracellular grains) of an early chick blastoderm (St. 5–6), after $[^{35}S]$sulphate incorporation. A section through the anterior half of the primitive streak is shown (see Fig. 1). The ventral side of the UL is positive. ML and DL show a diffuse label. Scale bar is 50 μm.

Cellular grain positivity is observed over ML and DL cells, but is nearly absent over the UL cells.

The positivity mentioned above, is largely removed after treatment with testicular hyaluronidase, a hydrolytic enzyme degrading hyaluronic acid, chondroitin sulphate A and C, and chondroitin (Fig. 2C, E). Thus, the positivity after tritiated glucosamine incorporation can probably be attributed to one or more of these products. Residual positivity is most pronounced where a basal lamina is present, indicating the synthesis of glycoproteins or testicular hyaluronidase-resistant AMPS (heparan sulphate or heparin, dermatan sulphate, keratan sulphate). However, we cannot exclude the presence of incompletely degraded hyaluronic acid or chondroitin sulphate A, C in a protecting matrix.

$[^{35}S]$sulphate incorporation resulted in a different distribution of silver grains

**Figure 2**

Autoradiographs (focused on extracellular grains) of an early chick blastoderm (St. 5–6), after $[^{3}H]$glucosamine incorporation. Sections through the anterior half of the primitive streak are shown.

(A) A general view. One half of the primitive streak and the lateral area of the embryo. Scale bar is 100 μm.

(B) Central region (see Fig. 1) after treatment with a buffer solution containing the heat-inactivated enzyme. Positivity is localized at the periphery of ingressing UL cells, ML and DL cells. In the groove, some label is present at the dorsal side of ingressing cells. Scale bar is 20 μm.

(C) Central region after testicular hyaluronidase pretreatment. A residual positivity is observed in ingressing UL cells, ML and DL cells. Scale bar is 20 μm.

(D) Lateral region (see Fig. 1) after treatment with a buffer solution containing the heat-inactivated enzyme. The ventral side of the UL and the periphery of the DL cells are positive. The apical surface of the UL is negative. Scale bar is 20 μm.

(E) Lateral region after testicular hyaluronidase pretreatment. The ventral side of the UL remains positive. The DL positivity is markedly reduced. Scale bar is 20 μm.
The cells of the UL, ML and DL did not show any preference for synthesis of sulphated products. In the extracellular compartment, an intense label was observed at the ventral side of the UL. The sulphated products were also present in the extracellular area of ML and DL.

Control experiments of blastoderms cultured (a) without the labelled precursors and (b) with the labelled precursors but at 4 °C, fail to show any positivity, indicating the absence of chemography or adsorptive effects during autoradiography.

**Measurement of labelled macromolecules released in the fixing medium**

The proportion of labelled macromolecules in the fixing medium, if compared with the labelled macromolecules in the blastoderms, is, for tritiated macromolecules, certainly less than 10%, and for sulphated macromolecules certainly less than 5%.

**Cellulose acetate electrophoresis**

The nature of the labelled products was further analysed using cellulose acetate electrophoresis. According to Breen et al. (1970), the LiCl/HCl medium allows a clear separation on the basis of the sulphate content/disaccharide ratio. We confirmed this by means of migration of standard AMPS. The hyaluronic acid (non-sulphated) remains near the origin, while heparin, showing a higher degree of sulphation than chondroitin sulphate A, B and C, migrates faster than the latter products (Fig. 4B).

The sample, containing labelled AMPS from blastoderms (Fig. 4A), shows three components: a tritium band near the origin, and two other bands, both containing tritium as well as $^{35}$S sulphate. The tritiated band (peak 1 in Fig. 4A) comigrates with reference hyaluronic acid. Although the synthesis of this product is probable, the possibility of the synthesis of non-sulphated chondroitin cannot be ruled out. The two double-labelled bands (peak 2 and 3 in Fig. 4A) migrate more slowly than the reference chondroitin sulphates. Consequently, those products show a lower sulphate content/disaccharide ratio than the reference chondroitin sulphates. This undersulphation does not seem to be the result of the procedure, because it was also observed by other authors using different techniques (Franco-Browder et al. 1973; Kvist & Finnegan, 1970; Manasek et al. 1973).

It is difficult to characterize AMPS with cellulose acetate electrophoresis alone, particularly since the components are undersulphated. Therefore, this technique was combined with the pretreatment of the sample with (1) nitrous acid, which degrades specifically $N$-sulphated AMPS, i.e. heparin sulphate or heparin (Inoue & Nagasawa, 1976), or (2) chondroitinase ABC, which degrades chondroitin sulphate A, B and C. The slowest double-labelled band (peak 2 in Fig. 4A) is sensitive to nitrous acid, and the second (peak 3 in Fig. 4A) to chondroitinase ABC.
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Fig. 4. Cellulose acetate electrophoresis in 0.05 M-LiCl/0.01 M-HCl. Potential gradient 10 V/cm for 45 min. (A) Separation of [3H]glucosamine (---) and [35S]sulphate (---) labelled AMPS of the early chick blastoderm. Peak 1: (3±1) mm; peak 2: (19±2) mm; peak 3: (27±2) mm. (B) A densitometer trace (629 nm) of a mixture of commercial hyaluronic acid, chondroitin sulphate A, B and C, and heparin.

Electrophoresis in Na-barbital gives a well-separated tritium band, coinciding with hyaluronic acid. The quality of separation of the sulphated components in the labelled sample was lower in the Na-barbital medium, compared with the LiCl/HCl medium.

DISCUSSION

The use of autoradiography in the study of the localization of AMPS relies on the efficient fixation of those macromolecules. Leaching of AMPS from cow...
uterus and vagina into the fixative (formalin-cetyltrimethylammonium bromide) did not appear to be more than 10% (Radmehr & Butler, 1978). Because Pousty, Bari-Kahan & Butler (1975) demonstrated that the solubility of AMPS in fixatives varies with the tissue source, the amount of solubilized labelled macromolecules from the blastoderms into the fixative was measured. Our results suggest that in chick blastoderms, the solubilization of AMPS into the fixative is negligible.

Tritiated glucosamine incorporation was carried out in order to study the extracellular AMPS. However, because glucosamine is a precursor of a wide variety of biological molecules (Warren, 1970), the labelled material had to be treated with testicular hyaluronidase, before processing for autoradiography. Our results reveal that the positivity of ML and DL cells largely disappears after this treatment. Because the testicular hyaluronidase preparations often contain some proteolytic activity, the loss of radioactivity in the autoradiographs could also be caused by the removal of glycoproteins from the sections, as tritiated glucosamine will label glycoproteins as well as glycosaminoglycans. Preliminary results of the separation of the labelled product in the papain digests, using gel chromatography, reveal that the proportion of tritiated low-molecular-size glycopeptides, if compared to the tritiated AMPS, is small. Therefore, synthesis of hyaluronic acid, chondroitin and chondroitin sulphate A and/or C may be assumed in ML and DL cells. Subsequently, evidence is provided for the synthesis of hyaluronic acid, chondroitin, and/or chondroitin sulphate A, C. Cinematographic observations (Vakaet, 1970) clearly demonstrate that ML and DL cells are actively migrating at this stage of development. The synthesis of this testicular hyaluronidase-sensitive fraction was also observed in the UL cells during their ingestion through the primitive streak, and their appearance in the ML. These autoradiographic results allow us to propose a correlation between the synthesis of testicular hyaluronidase-sensitive AMPS, and the migration of individual cells in the early chick blastoderm. The possible importance of AMPS, as components necessary for normal morphogenesis during this early stage of development, was also suggested by Solursh (1976). He proposed that the synthesis and secretion of hyaluronic acid, followed by extensive hydration (Ogston & Stanier, 1951), could result in the expansion of forming extracellular spaces. This cell-free space would then favour the migration of the ML and DL cells.

The incorporation of [35S]sulphate into macromolecular material has been used in this investigation as an assay for the synthesis of sulphated AMPS (for review see Kosher & Searls, 1973). However, the direct sulphation of proteins (Slomiany & Meyer, 1972) cannot be eliminated. The ubiquitous distribution of cells in the early chick blastoderm, which share the ability to synthesize these sulphated products, prove that this phenomenon is not a unique characteristic of cells undergoing chondrogenesis (for review see Abrahamsohn et al. 1975). It has also been suggested that all embryonic cells pass through a chondro-
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Acid mucopolysaccharides in early chick blastoderm (Holtzer & Matheson, 1970). However, in agreement with Kosher & Searls (1973), we feel it is more reasonable to assume that the synthesis of this material is necessary for normal morphogenesis during the earliest stages of development. Moreover, O'Hare (1973) demonstrated histochemically that qualitative as well as quantitative differences in AMPS occur with the onset of chondrification. The undersulphation of the sulphated AMPS, detected in the early chick blastoderm, could be one of these structural differences, and is currently under investigation in our laboratory.

The electrophoretical results clearly demonstrate the synthesis of non-sulphated and sulphated AMPS. Although the former contains the bulk of the tritium label, no conclusions about the total amount, or the rate of synthesis of AMPS can be made. In order to do this, knowledge of the specific activity of the labelled products, and of the precursor pools is necessary, which is very difficult because of the small amount of material available. In this study, we have limited ourselves to a consideration of the AMPS in the extracellular compartment. Other components of this compartment such as collagen (Johnson, Manasek, Vincon & Seyer, 1974), or glycoproteins (Manasek, 1975; Greenberg & Pratt, 1977; Mintz & Glaser, 1978) were not studied. The appearance of different AMPS in specific areas of the early blastoderm suggests an early specialization of the extracellular matrix.

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REFERENCES


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