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Regional specificity of glycoconjugates in *Xenopus* and axolotl embryos

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SUMMARY

This paper reviews work on the presence, synthesis and developmental regulation of glycoconjugates (proteoglycans, glycoproteins and glycolipids) in the early amphibian embryo.

In the axolotl there is little regional specificity of protein synthesis until the tailbud stage, but substantial regional specificity of glycoprotein synthesis from the end of gastrulation. Glycolipid synthesis is more uniform although a number of unique species are made in the epidermis.

Isolated explants from axolotl early gastrulae show three types of behaviour in terms of glycoprotein synthesis, corresponding to the classical germ layers. *Xenopus* embryos at this stage show a higher degree of mosaicism.

Changes of glycoprotein synthesis in response to mesodermal or neural induction follow the predicted course depending on the regional character of the induced tissue.

The regional binding patterns of a number of lectins and monoclonal antibodies specific for particular carbohydrate determinants are presented and their significance discussed.

INTRODUCTION

One of the main aims of our laboratory over the last few years has been to collect regional markers in the early embryo; a regional marker being a substance made only in a particular region, or a particular cell type. The reasons for this are twofold. Firstly, biochemical or immunological markers are necessary for the identification of cellular character following experiments on induction. They may enable earlier recognition of differentiated cell types than conventional histology and are particularly useful when the arrangement of tissue masses characteristic of the normal embryo has been deranged. Secondly, their biochemical nature may give us some clues about what is actually happening in early development and this particularly applies to markers which appear prior to terminal differentiation, or which do not correspond in regional distribution to terminally differentiated types.

In the search for markers we have paid special attention to glycoconjugates. Three classes of glycoconjugate are recognised in animal biochemistry and all are present either on the cell surface, or in the extracellular matrix or both. *Proteoglycans* are very large molecules composed of a core protein to which are

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linked side chains composed of repeating disaccharides, usually a hexuronic acid and an amino sugar. They are often sulphated. *Glycoproteins* form a rather miscellaneous group and the sugar content can range from a few percent to over 80 %. Oligosaccharides are either O-linked to serine or threonine, or N-linked to asparagine. A very wide variety of structures is possible although the N-linked types always contain mannose-rich core regions built up by the dolichol phosphate cycle. Glycoproteins which are integral components of the plasma membrane have a hydrophobic portion of the polypeptide chain which is inserted in the membrane, and a hydrophilic portion, to which the oligosaccharides are attached, projecting to the exterior. *Glycolipids* are composed of a long chain fatty alcohol, sphingosine, to which is attached a fatty acid and an oligosaccharide. Like membrane glycoproteins, glycolipids are retained in the plasma membrane by the hydrophobic part of the molecule and the oligosaccharide chain projects to the exterior.

All glycoconjugates, except for the mannose-rich cores of N-linked oligosaccharides, are formed by terminal addition of sugars catalysed by the appropriate glycosyl transferase. This is very important because it means that individual molecules displayed at the surface of one cell can tell neighbouring cells what combination of glycosyl transferases is being expressed. This seems the ideal molecular basis for an epigenetic code (Slack, 1980) and molecules identified in other systems as 'coding factors' have indeed proved to be glycoproteins (Trisler, Schneider & Nirenberg, 1981; Wilcox, Brower & Smith, 1981; Wilcox *et al.* 1984). Of course most glycoconjugates are probably not coding factors and have more mundane functions. Among these we can distinguish between functions which contribute to the terminally differentiated phenotype and functions which are concerned with things such as cell adhesion and cell movement and are thus likely to represent transient differentiations in the early embryo (Edelman, 1983). These are useful to us as regional markers but do not have the intrinsic importance of the coding factors.

The results described below represent to a large extent a preliminary survey since virtually no work has been carried out on this problem before. At the present state of knowledge we cannot be confident of having identified any coding factors but we have a number of examples of regional markers which are transient or terminal differentiation products.

BIOSYNTHESIS OF GLYCOPROTEINS IN THE AXOLOTL

Our initial studies were made using the axolotl. The embryos are larger than those of *Xenopus* hence easier to dissect accurately, and, more important, terminal differentiation commences in the various tissues rather later relative to morphological stage than in *Xenopus* (Mohun, Tilly, Mohun & Slack, 1980; Forman & Slack, 1980). This is advantageous because it gives us a greater chance of detecting the earlier, transient events. However, it has subsequently turned out that there are substantial differences in glycoprotein synthesis between axolotl, *Xenopus*

(Smith & Watt, 1985; Smith & Slack, unpublished) and *Pleurodeles* (Riou, Darribère & Boucaut, 1984), and this should be remembered when reading what follows.

Axolotl embryos incorporate rather little tritiated sugar until the end of gastrulation, and much of what is incorporated goes into lipids. From stage 14 onwards there is a substantial incorporation into glycoproteins which can be effectively separated on 4–8 % SDS gradient gels. Six major bands can be identified in the molecular weight range 80–500 kD. When early neurulae are dissected into regions corresponding to the visible tissue masses in the embryo it is evident that there is a considerable quantitative and qualitative variation in the molecules synthesized by the different regions (Slack 1984*a*, Fig. 1). This is in contrast to the picture obtained by studying incorporation of [^{35}S]methionine where the one-dimensional (1D) protein patterns show no regional variation (Fig. 2) and even two-dimensional (2D) patterns show little variation. We believe that the large protein synthesis

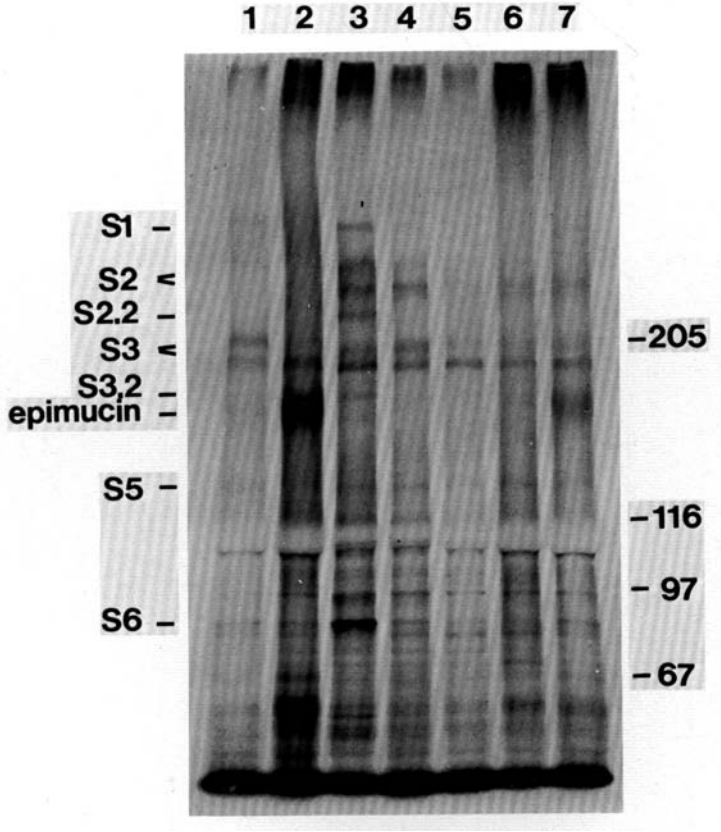


Fig. 1. Glycoprotein synthesis by crude membrane fractions from different regions of axolotl neurulae. Explants were labelled with tritiated galactose and mannose and equal amounts of protein run on a 4–8 % polyacrylamide gel. Track 1 neural plate, track 2 epidermis, track 3 notochord, track 4 dorsal mesoderm, track 5 ventrolateral mesoderm, track 6 archenteron wall, track 7 whole embryo.

differences seen in *Xenopus* embryos at comparable stages (Smith & Knowland, 1984) represent precocious terminal differentiation.

All the species S1–S6 are trypsin sensitive to at least some degree, and all bind to lentil lectin Sepharose and can be eluted with α-methyl mannoside. The species S2 has been identified as fibronectin by electrophoretic mobility and immunoprecipitation (Slack, 1985). This is made mainly although not exclusively by the mesoderm. The species S4 has been characterized by native molecular weight (470 kD) and sugar composition, and given the name 'epimucin' to reflect its specificity to the epidermis. It binds avidly to peanut lectin (see below).

BIOSYNTHESIS OF PROTEOGLYCAN AND GLYCOLIPIDS IN THE AXOLOTL

Proteoglycans appear as a smudge at the top of the gels used to analyse the glycoproteins. Incorporation of tritiated sugars into this 'high molecular weight polydisperse material' also increases sharply at the end of gastrulation although there is some at earlier stages. It is labelled by [^{35}S]sulphate but not by [^{35}S]methionine. Synthesis is concentrated in the yolky endodermal region of the neurula although there is some synthesis in all regions. The only identified

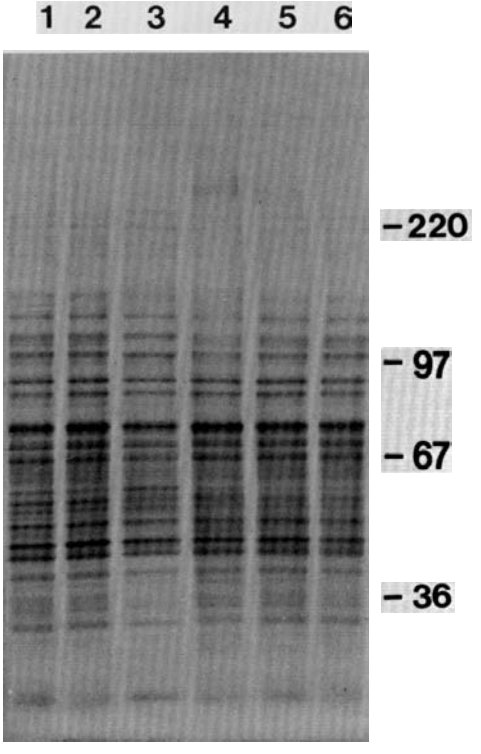


Fig. 2. Protein synthesis by different regions of the axolotl neurula. Explants were labelled with [^{35}S]methionine and equal c.p.m. were run on a 5–15 % polyacrylamide gel. Track 1 anterior neural plate, track 2 posterior neural plate, track 3 epidermis, track 4 notochord, track 5 dorsal mesoderm, track 6 ventrolateral mesoderm.

proteoglycan is keratan sulphate, which appears in the notochord during neurulation (Smith & Watt, 1985, and see below). An analysis of bulk proteoglycan(s) from early embryos of *Rana pipiens* had earlier showed them to be high molecular weight polyanions containing a variety of sugars and sensitive to pronase (Johnson 1977, 1978).

In contrast to the other glycoconjugates there is active synthesis of glycolipids from fertilization onwards. This is presumably associated with the requirement for large amounts of extra plasma membrane in the cleaving egg. The regional biosynthesis pattern of alkali-stable lipids (which are not necessarily all glycolipids) is shown in Fig. 3 and it is clear that there are no major regional differences outside the epidermis. The principal species was identified as galactocerebroside by chromatographic mobility, and by acid hydrolysis followed by chromatographic identification of the galactose (Slack 1984*a*).

BIOSYNTHESIS OF GLYCOPROTEINS IN EXPLANTS FROM GASTRULAE

We have studied the behaviour of small tissue explants taken from axolotl gastrulae at the beginning of gastrulation (Fig. 4) and cultured until controls reach the end of neurulation (Slack, 1984*b*, Cleine & Slack, 1985). The patterns of glycoprotein synthesis have been compared with those made by the structures which the regions studied would have become in the course of normal development. The fate map assignments are based on data from Smith & Slack (1983) and

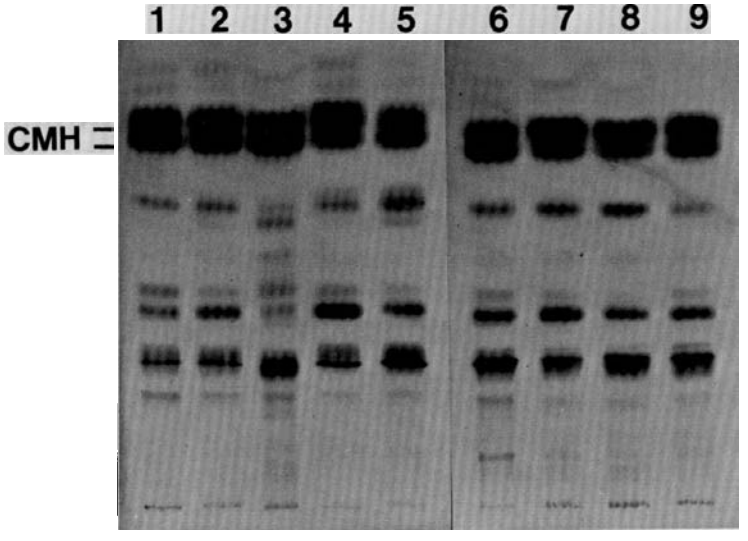


Fig. 3. Synthesis of alkali stable lipids by different regions of the axolotl neurula. Explants were labelled with [^{3}H]galactose and alkali stable lipids separated by thin-layer chromatography. CMH: galactocerebroside. Track 1 anterior neural plate, track 2 posterior neural plate, track 3 epidermis, track 4 notochord, track 5 dorsal mesoderm, track 6 ventrolateral mesoderm, track 7 archenteron wall, track 8 yolk mass, track 9 whole embryo.

Cleine & Slack (1985). All types of explant commenced glycoprotein synthesis on schedule, *i.e.* when controls finished gastrulation.

Animal pole region

In normal development this becomes mainly anteroventral epidermis. Explants began to synthesize epimucin, but also made more of the other species (S1,3,5,6 and fibronectin) than pure neurula epidermis. Studies of cilia and mucous granules by electron microscopy (Slack, 1984*b*) and of epimucin by peanut lectin binding (in *Xenopus*, Slack, 1985) show that most of the cells differentiating in an epidermal direction are on the exterior and only a few of the internal cells behave in this way. A similar result has been obtained in *Xenopus* with the epidermis-specific antibody 2F7C7 (Jones and Woodland, pers. comm.). By contrast all cells in this type of explant (from *Xenopus*) make epidermal-type keratin polypeptides (Dale, Smith & Slack, 1985) an antibody to which stains both inner and outer layers of the normal epidermis.

Vegetal pole region

In normal development this contributes to the endodermal yolk mass; much is probably extraembryonic since it ends up in the lumen of the gut and becomes digested during late development. The gastrula and neurula explants behave similarly: neither makes discrete glycoprotein species but both make the high molecular weight polydisperse material.

Dorsal marginal zone

This becomes pharyngeal endoderm, cephalic mesoderm and notochord. The notochord is characterized by a high level of fibronectin synthesis and by the presence of extra bands (S2.2 and S3.2). These features are found also in the DMZ explants although with a 'background' of polydisperse material. No epimucin is formed by these explants.

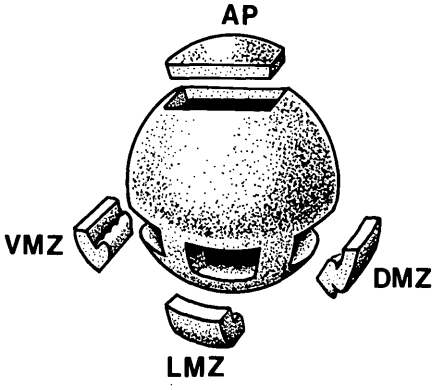


Fig. 4. Isolated regions from axolotl gastrulae used for the study of specification.

Ventral marginal zone

This becomes lateral plate mesoderm in the posterior half of the body with a small contribution to the somites. The explants also contained some prospective endoderm. The glycoprotein synthesis pattern was somewhat variable from experiment to experiment, sometimes resembling the animal pole region, sometimes the dorsal marginal zone, and sometimes intermediate.

Lateral marginal zone

This contributes to somites and lateral plate. The glycoprotein synthesis pattern resembled that of the dorsal marginal zone.

This study therefore showed that the five types of explant, all with distinctly different fates in normal development, fell into only three classes in terms of glycoprotein synthesis pattern. These correspond to the behaviour of the normal neurula tissues: epidermis, notochord and yolk mass, and it seems logical to identify these with the three germ layers, ectoderm, mesoderm and endoderm respectively. During the nineteenth century it was hotly debated whether the germ layers represented cell states or were merely descriptive terms (Oppenheimer, 1941). The ability to look at early differentiation events such as glycoprotein synthesis strengthens the case that they are indeed cell states.

It should be noted that the results of isolation experiments from early gastrulae of the axolotl are not the same as those from *Xenopus*. In the axolotl only three specified states are detected while in *Xenopus*, by histological criteria, there are probably five or six (Slack & Forman, 1980). The difference lies in the more highly mosaic character of the marginal zone. *Xenopus* does not lend itself to the kind of biosynthesis experiment described above, but one glycoconjugate, keratan sulphate, has been studied on sections of isolates using the monoclonal antibody MZ15 (see below) and it is clear that it only appears in DMZ and never in LMZ or VMZ explants (Dale *et al.* 1985).

CHANGES IN GLYCOPROTEIN SYNTHESIS OCCASIONED BY INDUCTION

Three experiments have been carried out to test whether the pattern of glycoprotein synthesis changes in accordance with the sequence of inductive interactions which we have proposed from histological data (Smith & Slack, 1983; Slack, Dale & Smith, 1984). Firstly, animal pole explants (axolotl) were treated with vegetalizing factor provided by Dr H. Tiedemann (Cleine & Slack, 1985). This produced a complete change in behaviour from epidermis-like to notochord-like, in other words the explants behaved as though they had been taken from the marginal zone rather than the animal pole region. This provides good evidence that this factor indeed has a 'mesodermalizing' effect on gastrula ectoderm.

Secondly, animal pole region explants were combined with DMZ explants (*Xenopus*), cultured until controls reached the end of neurulation, and then scored

for peanut lectin binding (Slack, 1985). PNA binding is very high for cement glands, positive for epidermis and negative for neuroepithelium. While isolated AP regions form only epidermis, the AP–DMZ combinations formed no epidermis but did form cement glands and neuroepithelium from the AP component. Cement glands can, on occasion, arise in *Xenopus* AP isolates but neuroepithelium never does so and so its appearance in these combinations can confidently be ascribed to neural induction.

Neural induction also occurs in the 'organizer' graft, following establishment of a secondary mesodermal axis (Smith & Slack, 1983; Slack *et al.* 1984). Examination of the secondary neural plate and neural tube showed that PNA binding was suppressed, as we would expect if these inductions are genuinely neuroepithelial in character (Slack, 1985; Smith, Dale & Slack, this volume).

USE OF ANTIBODIES AND LECTINS TO MAP THE REGIONAL DISTRIBUTION OF SPECIFIC CARBOHYDRATE GROUPINGS

The regional information provided by the biosynthetic studies is limited by the accuracy of the dissection, although they have the advantage that the immense background of reserve food materials inherited from the egg can be excluded. Fine grained regional studies must be carried out on histological sections and are thus necessarily studies of *total presence* of the substance in question rather than *current synthesis*. The following data is necessarily somewhat fragmentary since it represents the current state of research with the reagents which have become available to us. All the studies have been conducted by direct or indirect fluorescence using FITC conjugates, on TCA- or paraformaldehyde-fixed embryos embedded and sectioned in polyethylene glycol distearate (see Heasman, Wylie, Hausen & Smith, 1984). Most observations relate to *Xenopus* but where observations have been made on the axolotl they have been similar or identical, except where specified. The carbohydrate determinants which are known are shown in Fig. 5.

Lectins

Some lectins show uniform binding from early stages onwards. In this class are Concanavalin A which binds to mannose and glucose residues (Fig. 6) and wheat germ agglutinin which binds to β-D-GlcNAc. Some show enhanced binding to cell membranes and in particular to the membranes on the outer surface of the embryo. In this group are ricin (β-D-Gal), and *Dolichos biflorus* agglutinin (α-D-GalNAc) (Fig. 8). The outer membrane determinant is not retained in TCA-fixed material and is therefore presumably mainly polysaccharide in composition. It is probably the same as the i determinant (see below). *Ulex europaeus* lectin (α-L-Fuc) is negative at all stages until early larva when some binding to the gut lumen becomes apparent.

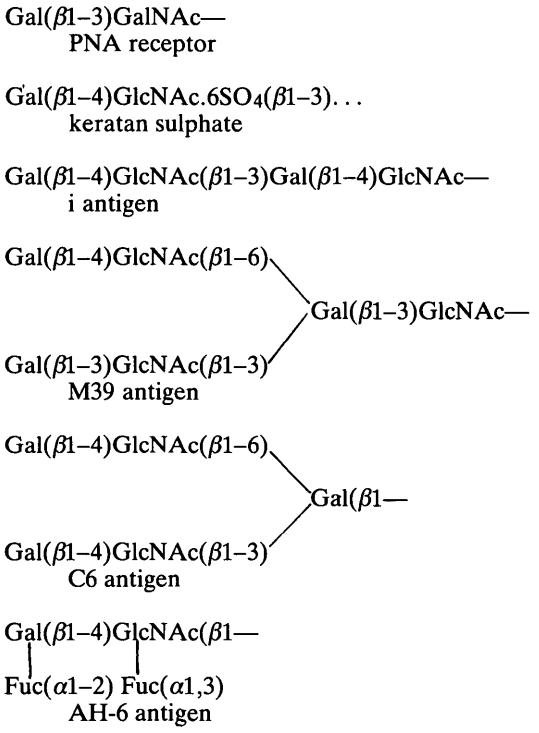


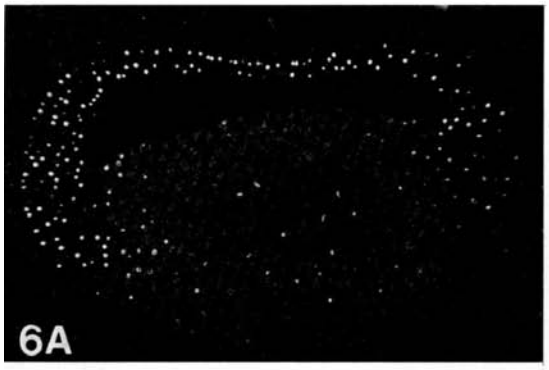
Fig. 5. Carbohydrate determinants recognised by the reagents used in this study.

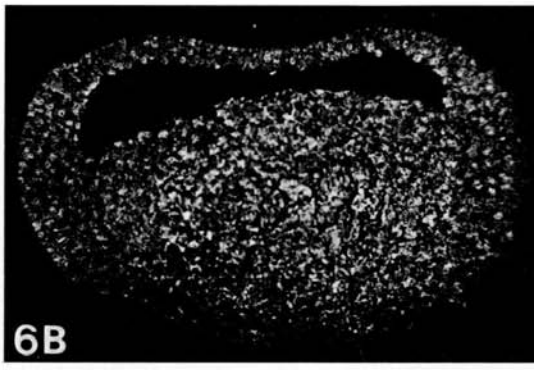
i autoantibody

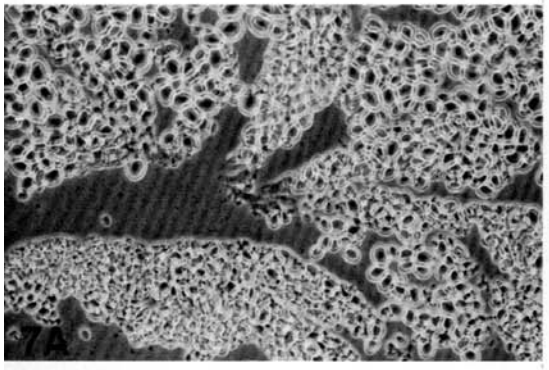
This is a human serum, provided by Dr T. Feizi (Clinical Research Centre, Harrow), which recognises the determinant D-Gal(β1–4)GlcNAc(β1–3)-Gal(β1–4)GlcNAc. This is present in the early embryo and also the oocyte, in which it is concentrated in the animal hemisphere and around the external surface. During cleavage a concentration also appears on internal membranes, although much less intense than on the exterior. The exterior label persists through gastrulation (Fig. 7) and in the neurula is found in the lumina of the neural tube and archenteron, known to be derived from the original surface layer (Smith & Malacinski, 1983). It also appears in the notochord sheath and in the notochord–neural tube junctional region. By the end of neurulation it has disappeared from the epidermis but persists on the internal luminal surfaces.

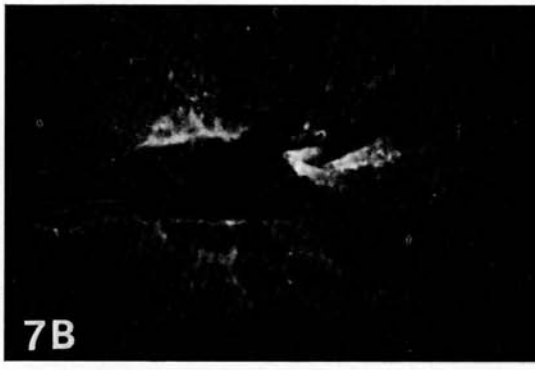
Fibronectin

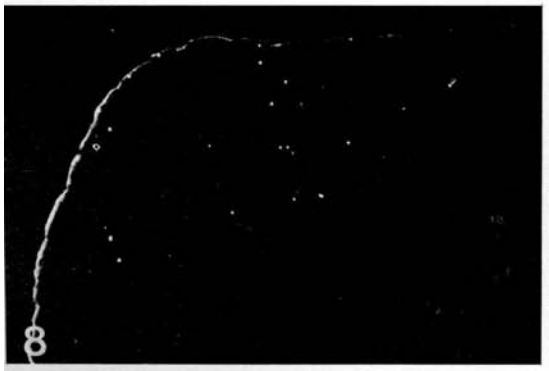
This was studied using an antibody donated by Dr C. Wylie (St. Georges). Like other workers, we have found small amounts of fibronectin around all the cells of the gastrula, and particularly lining the blastocoelic cavity (Fig. 9, and Lee, Hynes & Kirschner, 1984; Boucaut & Darribère, 1983). From the onset of neurulation bright fluorescence was apparent in all parts of the extracellular matrix (Fig. 10).

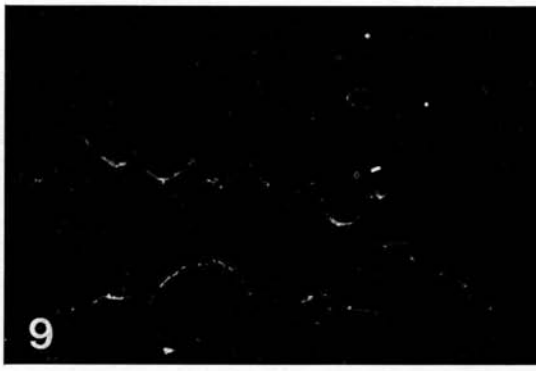


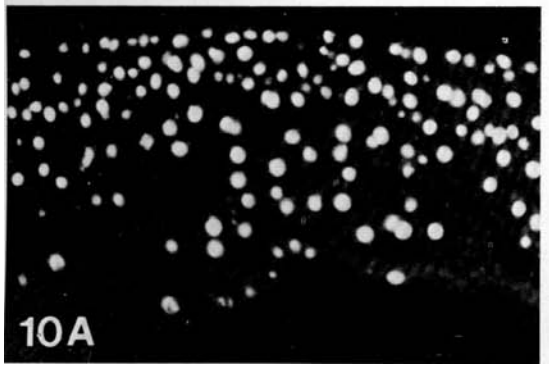




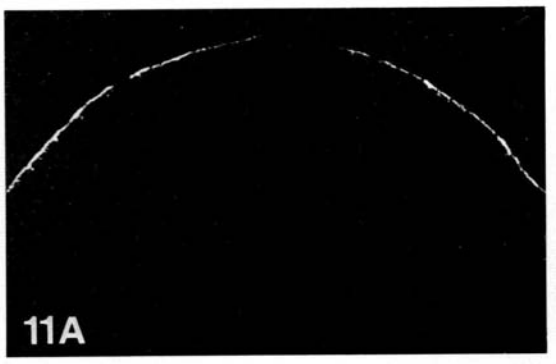








Figs 6–10



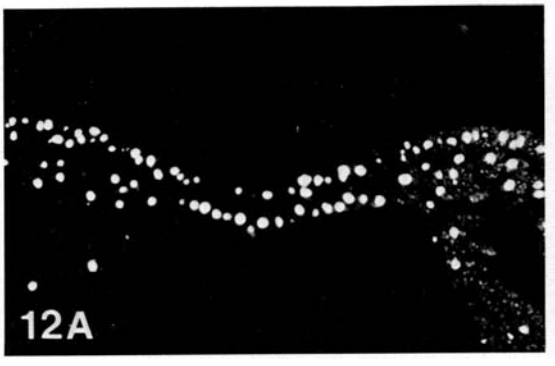


Fig. 11. Peanut lectin receptors in a stage-20 *Xenopus* embryo. (A) FITC-PNA binding, (B) The same after neuraminidase treatment.
Fig. 12. Binding of the 4H8 monoclonal antibody to the neural plate of an axolotl neurula. (A) DAPI fluorescence, (B) 4H8.

Arachis hypogaea (*peanut*) *lectin*

This is specific for the grouping D-Gal (β1–3)GalNAc. Early embryos are uniformly negative but from the commencement of neurulation binding becomes apparent in the future epidermis (Fig. 11A). Later, a little binding occurs in the extracellular matrix particularly between the notochord and neural tube. After treatment of the sections with neuraminidase the entire extracellular matrix becomes very bright (Fig. 11B), showing that the matrix receptor predominantly carries the grouping: NANA-Gal(β1–3)GalNAc. Studies using affinity

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Fig. 6. Con A receptors in *Xenopus* early gastrula. (A) DAPI fluorescence, (B) FITC Con A binding.
Fig. 7. i determinant in the bottle cells in a *Xenopus* middle gastrula. (A) phase contrast, (B) antibody binding.
Fig. 8. Receptor for *Dolichos biflorus* lectin in a *Xenopus* early gastrula.
Fig. 9. Fibronectin in the *Xenopus* blastocoel cavity.
Fig. 10. Fibronectin in the extracellular matrix of a *Xenopus* early neurula. (A) DAPI fluorescence (B) anti-fibronectin. Note that the antibody also binds to the vitelline membrane but not to the external embryo surface.

chromatography and immunoprecipitation have shown that in the axolotl the epidermal receptor is carried by epimucin and the matrix receptor by a variety of species including fibronectin (Slack, 1985).

M39

This is a monoclonal antibody, provided by Dr T. Feizi (Clinical Research Centre, Harrow), and has the specificity shown in Fig. 5. It also binds to the extracellular matrix, like neuraminidase/PNA and anti-fibronectin.

4H8

This is a monoclonal antibody made by Dr E. Jones (Warwick University) against a preparation of axolotl embryo glycoproteins from our laboratory. Early stages are negative and from the end of gastrulation it appears in the developing neuroepithelium (Fig. 12). Not every cell is labelled but many are, suggesting some heterogeneity of cellular character in the neural plate. Most of the epidermis is negative although a few cells outside the neural folds sometimes appear labelled. This antibody is species specific, reacting with the axolotl but not with *Xenopus*, and the nature of the immunological determinant is not yet known.

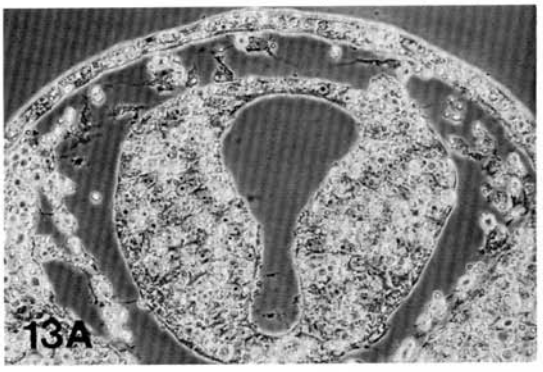
NC1

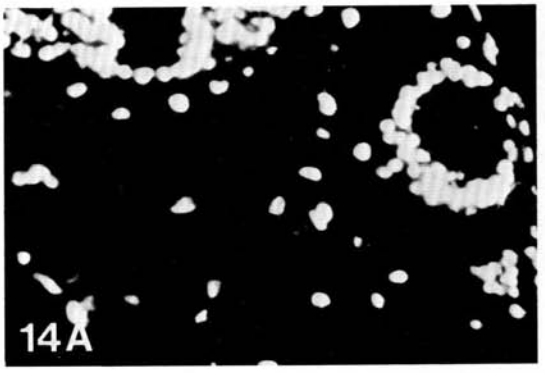
This is a monoclonal antibody specific for neural crest cells in the chick embryo (Vincent & Thiery, 1984). It was provided by Dr G. Tucker (Nogent sur Marne). It is thought to react with the carbohydrate part of a glycoprotein but the structure of the determinant is not yet known. In *Xenopus* it does not react with early stages but stains the fibre tracts in the neural tube when these appear at the mid tailbud stage (Fig. 10). Later, it also stains enteric ganglia and associated axonal processes.

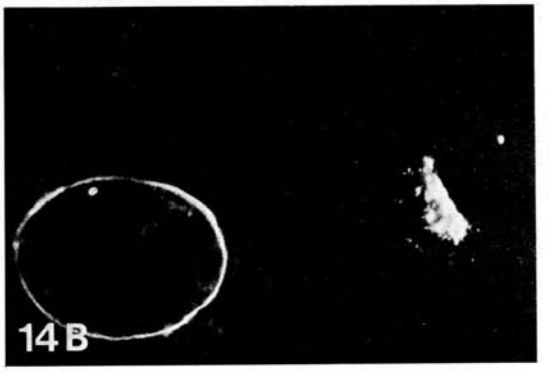
MZ15

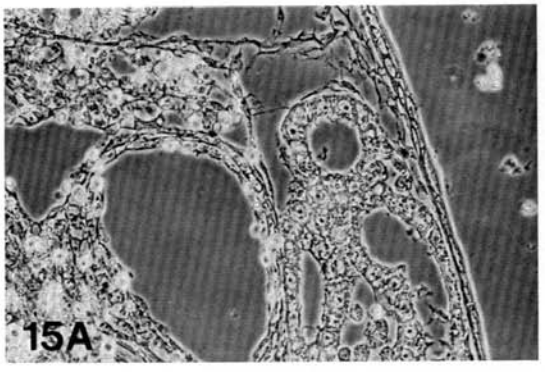
This is a monoclonal antibody specific for the proteoglycan keratan sulphate. The polysaccharide chains in this species consist of the repeating disaccharide Gal(β1–4)GlcNAc(6SO_4)(β1–3) but in some respects keratan sulphate resembles a glycoprotein rather than a typical proteoglycan since it has a GlcNAc-Asn glycopeptide linkage. The antibody does not react with early stages but from mid-neurula it stains the notochord: principally the outer sheath but also the

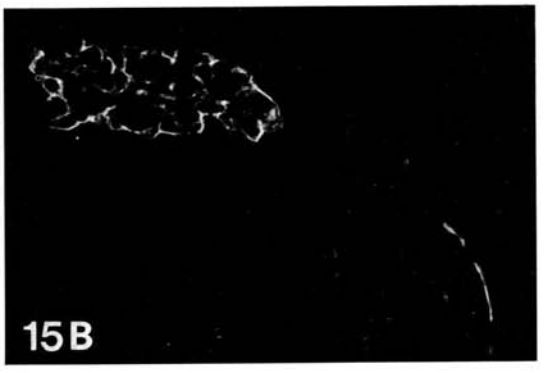
Fig. 13. Binding of NCl monoclonal antibody to the fibre tracts of the neural tube in a stage-32 *Xenopus* embryo. (A) phase contrast, (B) NCl.
Fig. 14. Binding of MZ15 monoclonal antibody to a stage-40 *Xenopus* prelarva. The antibody binds to the notochord and the ventral part of the ear vesicle. (A) DAPI fluorescence, (B) MZ15.
Fig. 15. Binding of C6 monoclonal antibody to the mesonephros and pronephros of a stage-41 *Xenopus* pre-larva. (A) phase contrast, (B) C6.
Fig. 16. Binding of AH6 monoclonal antibody to the pharynx of a stage-41 *Xenopus* pre-larva. (A) DAPI fluorescence, (B) AH6.

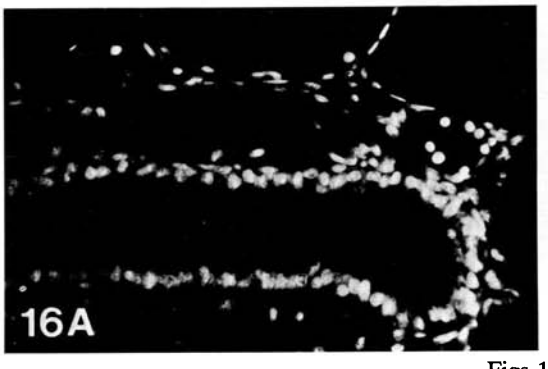












Figs 13–16

developing chordocytes (Smith & Watt, 1985 and Fig. 14). Staining is increased if the sections are treated with chondroitinase. In the tailbud stage the ventral part of the auditory vesicle, an epidermal derivative, is also stained. When the vertebral cartilages develop, in the tadpole, the notochord becomes negative.

C6

This is a monoclonal antibody directed against the determinant shown in Fig. 5 and was provided by Dr B. Fenderson (Seattle). It does not react at all with early stages, but from the tailbud stage onwards it stains the mesonephros and the lumina of the pronephric tubules of sections pretreated with neuraminidase (Fig. 15).

AH-6

This is another monoclonal provided by Dr B. Fenderson and reacts with the determinant shown in Fig. 5 (the 'Y hapten'). It stains the gut lumen in prelarval stages, particularly the lining of the oropharynx (Fig. 16).

DISCUSSION

Most of the studies reported here clearly belong in the province of descriptive rather than experimental embryology. However this is inevitable in a preliminary survey and the isolation and induction experiments have indicated that the glycoconjugates are reliable markers of embryo regions and can be used to assess cellular character at an earlier stage than the traditional histological methods. Immunofluorescence of these regional markers can be a particularly powerful and elegant method when combined with the use of a fluorescent lineage label, visualized through a separate channel (see Smith *et al.* this volume).

Reasoning from the stage and regional distribution of the molecules and determinants studied we can roughly classify them into transient and terminal differentiation products as follows:

Transient	*Terminal*
Fibronectin	Epimucin
M39 receptor	NC1 receptor
Ricin/DBA/i receptor	C6 receptor
4H8 receptor	AH-6 receptor
keratan sulphate (notochord)	UEA receptor
	keratan sulphate (cartilage)

The information we have about functions is still scanty. We know that *Bufo* embryos exogastrulate when grown in concentrations of tunicamycin sufficient to inhibit N-linked glycoprotein synthesis (Sanchez & Barbieri, 1983). We also know that gastrulation movements in *Pleurodeles* can be inhibited by antibody to fibronectin injected into the blastocoel (Boucaut *et al.* 1984, and this volume).

It is probable that none of the determinants listed here are coding factors since they appear too late and the regional distribution is not what we would expect from embryological experiments designed to reveal the codings. However there are some regionalizations which do not correspond to traditional anatomical subdivisions. In particular we may note the i determinant and the non-sialylated PNA matrix receptor between archenteron roof and neural plate; and keratan sulphate on the ventral face of the auditory vesicle lumen. Whether such regions are part of the 'second anatomy' as originally proposed (Slack, 1982) must await further experiments.

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DISCUSSION

Speaker: J. Slack:

Question from J. Gurdon (Cambridge):
You show extracellular material stained with the antibody to fibronectin: I take it you can't really be sure where that has come from? Do you think it comes from any particular cell type?

Answer:
Several groups have shown that all the cells of early embryos make fibronectin. Fibronectin is, in fact, the band S2 on the gel in Figure 1. Per unit of protein there is more new synthesis in the notochord and the somites than elsewhere, but there is some synthesis all over the embryo.