

Changes in the expression of the carbohydrate epitope HNK-1 associated with mesoderm induction in the chick embryo

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Summary

We report that a monoclonal antibody, HNK-1, identifies specific regions and cell types during primitive streak formation in the chick blastoderm. Immunohistochemical studies show that the cells of the forming hypoblast are HNK-1 positive from the earliest time at which they can be identified. Some cells of the margin of the blastoderm are also positive. The mesoderm cells of the primitive streak stain strongly with the antibody from the time of their initial appearance. In the epiblast, some cells are positive and some negative at pre-primitive-streak stages, but as the primitive streak develops a gradient of staining intensity is seen within the upper layer, increasing towards the primitive streak. At later stages of development, the notochord and the mesenchyme of the headfold are positive, while the rest of the mesoderm (lateral plate) no longer expresses HNK-1 immunoreactivity. This antibody therefore reveals changes associated with mesodermal induction: before induction, it recognizes the 'inducing' tissue (the hypoblast) and reveals a mosaic pattern in the responding tissue (the epiblast); after

primitive streak formation, the mesoderm of the primitive streak that results from the inductive interactions expresses the epitope strongly.

Affinity purification of HNK-1-related proteins in various tissues was carried out, followed by SDS-PAGE to identify them. The hypoblast, mesoderm and epiblast of gastrulating chick embryos have some HNK-1-related proteins in common, while others are unique to specific tissues. Attempts have been made to identify these proteins using Western blots and antibodies known to recognize HNK-1-related molecules, but none of the antibodies used identify the bands unique to any of the tissues studied. We conclude that these proteins may be novel members of the HNK-1/L2 family, and that they may have a role in cell interactions during early development.

Key words: gastrulation, primitive streak, induction, mesoderm, hypoblast, chick embryo, morphogenesis, HNK-1 antibody, glycoconjugates, embryonic axis.

Introduction

The process of gastrulation results in the formation of the three primary germ layers and the establishment of the embryonic axis. In amniotes, the third of the layers to arise, the mesoderm, first condenses as a structure known as the primitive streak, which marks the future embryonic axis; its appearance represents the earliest visible indication of bilateral symmetry in the embryo. Formation of the primitive streak, at the future caudal end of the embryo, involves a change in morphology of some of the cells in the upper layer (epiblast), which lose their epithelial character and become a loose mesenchyme (for review see Bellairs, 1986).

Neither the mechanisms that trigger the epithelium-to-mesenchyme conversion nor the factors that determine which cells will undergo this transformation and which will not are understood. It is generally accepted, however, that the early lower layer, the hypoblast, exerts an 'inductive' influence on the overlying epiblast to trigger certain cells in the latter tissue to change their developmental fate. This view is based mainly on the pioneering experiments of Waddington (1930, 1933), who found that after rotating the hypoblast by 180° about its rostrocaudal axis, the primitive streak formed at the original rostral end of the embryo. Later, other workers extended and confirmed Waddington's original experiments (e.g. Azar & Eyal-Giladi, 1979, 1981;

Mitrani & Eyal-Giladi, 1981; Mitrani *et al.* 1983). While these results make it clear that the hypoblast plays an important role in formation of the primitive streak, it is still unknown whether the influence of the hypoblast is a true, *inductive*, induction (as defined by Slack, 1983; see also Gurdon, 1987). The answer to this question will have to come from single cell studies and marking experiments, and it would be helpful to have markers that recognize specific regions or cell types during very early development.

Lack of a reliable marker that can detect tissue phenotypes as they arise has hampered progress in the understanding of streak formation. Some undertakings have been made to find tissue differences in expression of proteins (Wolk & Eyal-Giladi, 1977; Cook *et al.* 1979; Zalik *et al.* 1987) and carbohydrate antigens (Thorpe *et al.* 1988), but none have as yet demonstrated any relation to the initiation of streak formation. Various adhesion molecules have been shown to be regulated during streak formation including N-CAM (Keane *et al.* 1988), A-CAM (Duband *et al.* 1988) and the cadherins (Hatta & Takeichi, 1986; Takeichi, 1988). However, their pattern of expression suggests that they are a result, rather than the cause, of the epithelium-to-mesenchyme conversion.

In this report, we describe a molecular marker, the HNK-1 epitope, exhibiting a temporal and spatial distribution that can be related closely to the morphogenesis of tissues involved in the establishment of the craniocaudal axis.

Materials and methods

Immunohistochemistry

Chick blastoderms were obtained from eggs incubated at 38°C for 4 to 24 h, until they had reached between stages X (pre-primitive-streak stages in Roman numerals according to Eyal-Giladi & Kochav, 1976) to stage 6 (post-primitive-streak stages in Arabic numerals according to Hamburger & Hamilton, 1951). The blastoderms were removed from the yolk and vitelline membrane and fixed in absolute ethanol for 24 h (for immunofluorescence) or 4% buffered formal saline (pH 7.0) for 1 h (for immunoperoxidase).

Embryos destined to be sectioned were embedded in gelatin. After washing three times in phosphate-buffered saline pH 7.6 (PBS), the blastoderms were placed in 5% sucrose-PBS for 3 h followed by 15% sucrose-PBS for 24 h at 4°C. They were then placed in the embedding medium, which consisted of 7.5% gelatin (300 bloom, Sigma) in 15% sucrose-PBS at 38°C for 1 h; the medium was then allowed to gel at room temperature before storing at 4°C. The blocks were then trimmed and quickly frozen in isopentane that had been cooled on dry ice. Sections were cut at 8–10 µm at –20°C on Bright cryostat, mounted on gelatin-subbed slides and air-dried, after which they were stored desiccated at 4°C.

For immunoperoxidase histochemistry, the sections were washed in warm PBS to remove the gelatin, and incubated for 1 h at room temperature in 2% bovine serum albumin (BSA, Sigma) in PBS to block nonspecific binding. The blocking solution was replaced with supernatant from HNK-1 myeloma cells (Abo & Balch, 1981) diluted 1:10 in PBS containing 0.1% BSA. After 1 h, the sections were washed in three changes of PBS and incubated for 1 h at room temperature with affinity-purified goat anti-mouse IgM-peroxidase conjugate (Sigma) diluted 1:100 in PBS containing 0.1% BSA. The sections were then washed in PBS three times before developing in a 0.5 mg ml⁻¹ solution of 3,3',5,5'-diaminobenzidine tetrahydrochloride (Aldrich) in 0.1 M-Tris-HCl (pH 7.6) containing 0.3% H₂O₂ for 10 min, followed by washing in tap water. The slides were then mounted in Aquamount (BDH) before viewing. Whole-mount blastoderms were stained by the same procedure directly after being fixed.

For immunofluorescence, blastoderms were fixed in absolute ethanol for 24 h before embedding as above. Following incubation with HNK-1 supernatant as described previously, the sections were incubated at room temperature for 1 h in the dark with goat anti-mouse IgM FITC conjugate (Sigma). After washing in PBS, the slides were mounted in a nonquenching medium (14% Gelvatol 20/30 [Fisons] containing 8.5 mg ml⁻¹ diazobicyclo-octane [DABCO, Aldrich, the anti-quenching agent], 30% glycerol and 350 µg ml⁻¹ sodium azide as preservative in a PBS base, pH 6.8). The slides were viewed on an Olympus Vanox T microscope equipped with epifluorescence optics, excited by a 200 W high pressure mercury vapour lamp at 405–455 nm (blue), using filters to allow emission at 460–520 nm (green).

Affinity extraction with immobilized HNK-1

Various tissues were dissected from unfixed blastoderms in PBS containing 1 mM-PMSF and frozen with solid CO₂. Epiblast was obtained from stage XIV embryos, hypoblast from stage-2 blastoderms and primitive streak from stage-2 to -3 embryos. Each sample was then dissolved in solubilization buffer, containing 0.25% deoxycholate, 20 mM-Tris, 150 mM-NaCl, 1 mM-EDTA, 1 mM-EGTA (pH 8.3). The detergent extract was then cleared by centrifugation and the supernatant incubated with a column of purified HNK-1 IgM coupled to CNBr-activated Sepharose 6B (Pharmacia) according to the manufacturers' protocol. 3 mg of purified HNK-1 IgM were coupled per ml of agarose. The column was washed with twenty column volumes of solubilized buffer containing 300 mM-NaCl. Bound protein was eluted with 0.1 M-diethylamine, 0.1% deoxycholate, 150 mM-NaCl, 1 mM-EDTA, 1 mM-EGTA (pH 11.5). Protein fractions were concentrated by pressure dialysis into 20 mM-Tris, 0.1% deoxycholate, 0.15 M-NaCl (pH 8.3).

Protein estimations were performed according to Bramhall *et al.* (1969); 3 µl of sample from each tube were applied to a 1 cm square of Whatman 50 hardened filter paper, which was then fixed in a 5:1:4 solution of methanol, acetic acid and deionized water, stained for 30 min in a 4:2:4 solution of methanol, acetic acid and deionized water.

containing 0.08% Coomassie brilliant blue R250 (Sigma), rinsed in tap water, destained in 7.5% methanol and 5% acetic acid in deionized water, dried and then placed in an Eppendorf tube. 1 ml of 66% methanol/1% ammonia was added to the tubes, which were then vortex mixed for 10 s. 15 min later, the absorbance (590 nm) of the protein solutions was determined and read from a linear plot of concentration of standards. Finally, an appropriate volume of sample buffer (see below) was added to each tube to standardize the protein concentration of all samples.

SDS-Polyacrylamide gradient minigels (prepared according to Matsudaira & Burgess, 1978) were used for protein separation of affinity-purified samples. Following the method of Laemmli (1970): 1 µg of protein was separated on 4–15% gradient SDS-polyacrylamide gels (200 V, constant voltage) under reducing (50 mg ml⁻¹ dithiothreitol; BDH) or nonreducing conditions for 1.5–2 h. Molecular weight markers (Rainbow Markers, Amersham, range 14.3–200 × 10³ M_r) were run on a parallel lane under reducing or nonreducing conditions. The electrode buffer used was SDS-glycine-Tris (pH 8.3), and samples were applied in SDS-glycerol-Tris (pH 6.8) containing 500 µg ml⁻¹ bromophenol blue.

The gels used for visualizing the HNK-1-related proteins were stained with silver by the following method: after fixation in 10% acetic acid/50% ethanol for 15–30 min, they are placed in 5% acetic acid/7.5% ethanol for 15–30 min. The gels were then transferred in 10% glutaraldehyde for 15 min to fix the proteins, washed extensively with distilled water, placed in 5 µg ml⁻¹ dithiothreitol for 20 min, incubated for 15 min in 0.1% AgNO₃ and developed for 10 min in 3% Na₂CO₃ containing 0.05% formalin. The reaction was stopped with 2.3 M-citric acid.

For immunoblotting, separated proteins were transferred electrophoretically to PVDF membranes (Immobilon, Millipore) for 1.5 h at 100 mA using the Novablot multiphor system (LKB), using 39 mM-glycine, 48 mM-Tris, 0.0375% SDS and 20% methanol. A portion of each membrane was stained immediately with Aurodyne (Janssen) to check for even transfer of protein, and the remainder blocked for a minimum of 12 h at 4°C with 5% BSA in 20 mM-Tris containing 0.9% NaCl and 20 mM-sodium azide (pH 8.2) (Tris-saline pH 8.2). The membranes were then incubated with monoclonal or polyclonal antibodies against known adhesion molecules and against other molecules considered to be likely candidates. These included monoclonal and polyclonal antibodies directed against N-CAM (supplied by Dr Urs Rutishauser), G4 antigen (supplied by Dr Fritz Rathjen), integrin (JG22, obtained from the Developmental Studies Hybridoma Bank, originally raised by Dr David Gottlieb) and tenascin (obtained from the Developmental Studies Hybridoma Bank, originally raised by Dr Douglas Famborough). Primary antibodies were diluted in 0.1% BSA/Tris-saline pH 8.2, and incubated at room temperature for 1 h (or 12 h at 4°C in cases where the affinity of the antibody was considered to be low) with continuous agitation. The binding of primary antibodies was detected using the appropriate Auroprobe-BLplus (Janssen) secondary antibody with silver enhancement, according to the manufacturers' protocol. All incubations were followed by three 5 min washes in 0.1% BSA/Tris-saline pH 8.2.

Results

Immunohistochemistry

HNK-1 expression during formation of the hypoblast

The HNK-1 epitope is already present at stage X, on the cells of the forming hypoblast. Using either immunoperoxidase or immunofluorescence, HNK-1-positive cells can be seen in close association with the lower surface of the epiblast (Fig. 1). These cells are larger than those of the epiblast and appear to be yolk-laden. At stage X–XI, HNK-1-positive cells are also seen within the epiblast (Fig. 2). In sections viewed by immunofluorescence, most cells are seen to contain intracellular staining in the form of a single, localized, bright region (Fig. 2). However, the cell surface is also stained. The surface of the cells of the hypoblast stains with increased intensity as these cells coalesce into a sheet reaching a peak when the hypoblast is fully formed at stage XIV. When hypoblast formation is complete, by late stage XIV, the entire hypoblast sheet is homogeneously stained with HNK-1 (Fig. 2).

Coincidental with the completion of hypoblast formation, the basal lamina underlying the epiblast stains strongly with HNK-1 (Fig. 3A). As the hypoblast sheet is replaced with the definitive (presump-

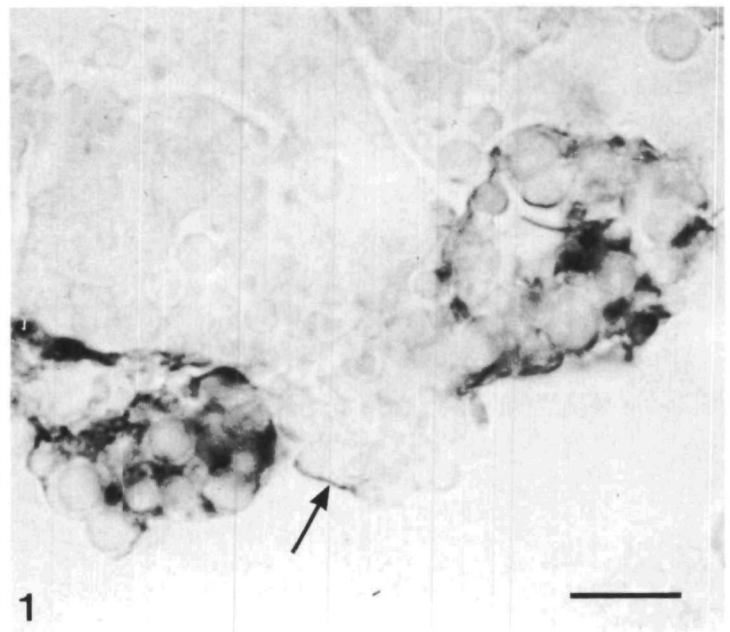


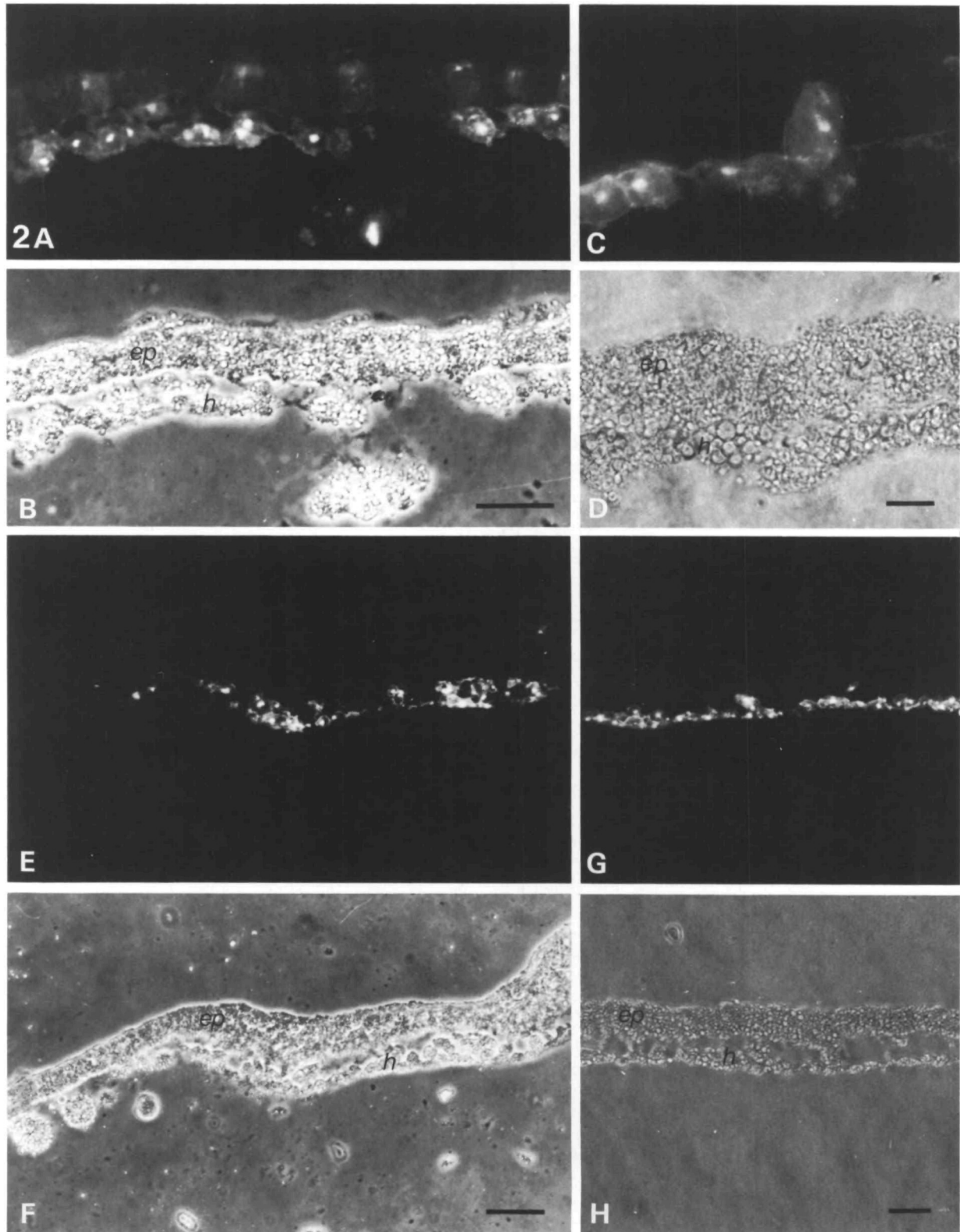
Fig. 1. Immunoperoxidase staining of st. X blastoderm. The photograph shows two HNK-1-positive cells associated with the ventral aspect of the epiblast. Between them lies what appears to be a protruding epiblast cell which, although not expressing HNK-1 all over its surface, displays a small area (arrow) of HNK-1 positivity. Bar, 10 µm.

tive gut) endoderm, the hypoblast remains HNK-1 positive as it moves to assume a position close to the anterior margin of the *area pellucida*.

Germ wall (area opaca endoderm)

At the same time as the first appearance of HNK-1-

positive cells beneath the epiblast, a few large cells in the posterior margin of the germ wall (*area opaca* endoderm; the 'marginal zone', Azar & Eyal-Giladi, 1979) also express HNK-1. This pattern persists up to the mid-primitive-streak stage (Fig. 3). These cells appear to bear many processes, which are in contact



with other HNK-1-positive cells of the posterior marginal zone. It is rare to see an isolated HNK-1-positive cell in this region. After examining many sections of early stage blastoderms, we have localized these exclusively to a narrow crescent in the posterior margin of the germ wall of the embryo. In some instances, this region of staining can be seen to be in intimate contact with the hypoblast (e.g. Fig. 3A,B). The anterior and lateral margins of the germ wall contain no cells bearing the HNK-1 epitope, nor do deeper regions of the *area opaca* endoderm.

HNK-1 expression during formation and regression of the primitive streak

As soon as a primitive streak can be detected at stage 2, the cells in this structure stain strongly with HNK-1 (Fig. 3B). As the streak develops, all its cells express the HNK-1 epitope, with the exception of some cells (Fig. 3D), which because of their position in the primitive streak are probably the precursors of definitive (gut) endoderm cells destined to form the new lower layer (Stern & Ireland, 1981). As the primitive groove forms in the centre of the streak at stage 3+, HNK-1 positivity of the cells of the mesoderm of the forming lateral plates is observed (Fig. 4). The cells of the primitive streak contain a single bright spot of fluorescent staining similar to that seen during hypoblast formation (c.f. Fig. 2 with Fig. 4). As the embryo develops, the staining intensity of the cells of the primitive streak increases (Fig. 3).

By the full ('definitive') primitive streak stage (st. 4), HNK-1 positivity is gradually lost from the mesoderm, with only a few cells remaining positive at the midline of the primitive streak (Fig. 4).

As Hensen's node regresses and the primitive streak shortens, the cells of the condensing notochord stain with HNK-1 (Fig. 5A). These cells exhibit

intracellular spots of staining (Fig. 5A) similar to those seen during hypoblast formation. The mesenchyme of the head fold is also HNK-1 positive (Fig. 5B,C).

HNK-1 expression in the epiblast

Prior to primitive streak formation the epiblast manifests a variegated, low intensity, staining pattern with HNK-1. At stage XIII, some (about 35%) epiblast cells exhibit HNK-1 staining on their surface. Indeed, if the epiblast is viewed from above in a whole-mount preparation, a striking 'mosaic' pattern is observed (Fig. 6). This pattern is seen throughout the surface of the *area pellucida* epiblast. The size of the cells staining with HNK-1 in the epiblast is quite variable (Fig. 6B), and their proportion is high; the pattern is therefore unlikely to be due entirely to noningressed hypoblast cells.

At the onset of gastrulation (st. 2), the cells of the epiblast overlying the primitive streak stain intensely with HNK-1, while the rest of the epiblast shows an anteroposterior gradient in staining, with increasing intensity from the anterior margin of the *area pellucida* to the primitive streak (Fig. 3B,C).

Affinity purification of HNK-1 related proteins from the embryo

Purified monoclonal antibody HNK-1 IgM coupled to CNBr-activated Sepharose was used to isolate HNK-1-related proteins from st. XIV epiblast, st. 2-3 primitive streak and st. 2 hypoblast. The results are illustrated in Fig. 7, and summarized in Table 1.

Epiblast

The dominant component of the column eluate of epiblast was a high relative molecular mass [M_r more than 300×10^3 (300K)] glycoprotein complex, which, on reduction, fractionated into many peptides. Because of the high degree of glycosylation, which resulted in a 'smeared' appearance (Fig. 7A), the molecular weight of this complex, which is unique to the epiblast, proved difficult to measure under non-reducing conditions. A second major component is a triplet of 78, 71, 68K, the latter band being the most prominent. In addition, a minor band at 105K was also detected, as were diffuse, lightly stained, bands at 55 and 41K. Under reducing conditions the most abundant polypeptide fragment has an apparent M_r of 144K, with many less-intensely stained bands of lower molecular weight.

Primitive streak

A prominent band of 32K characterizes the primitive streak sample, the position of which does not alter under reducing conditions. The eluate from the primitive streak sample also contained some bands

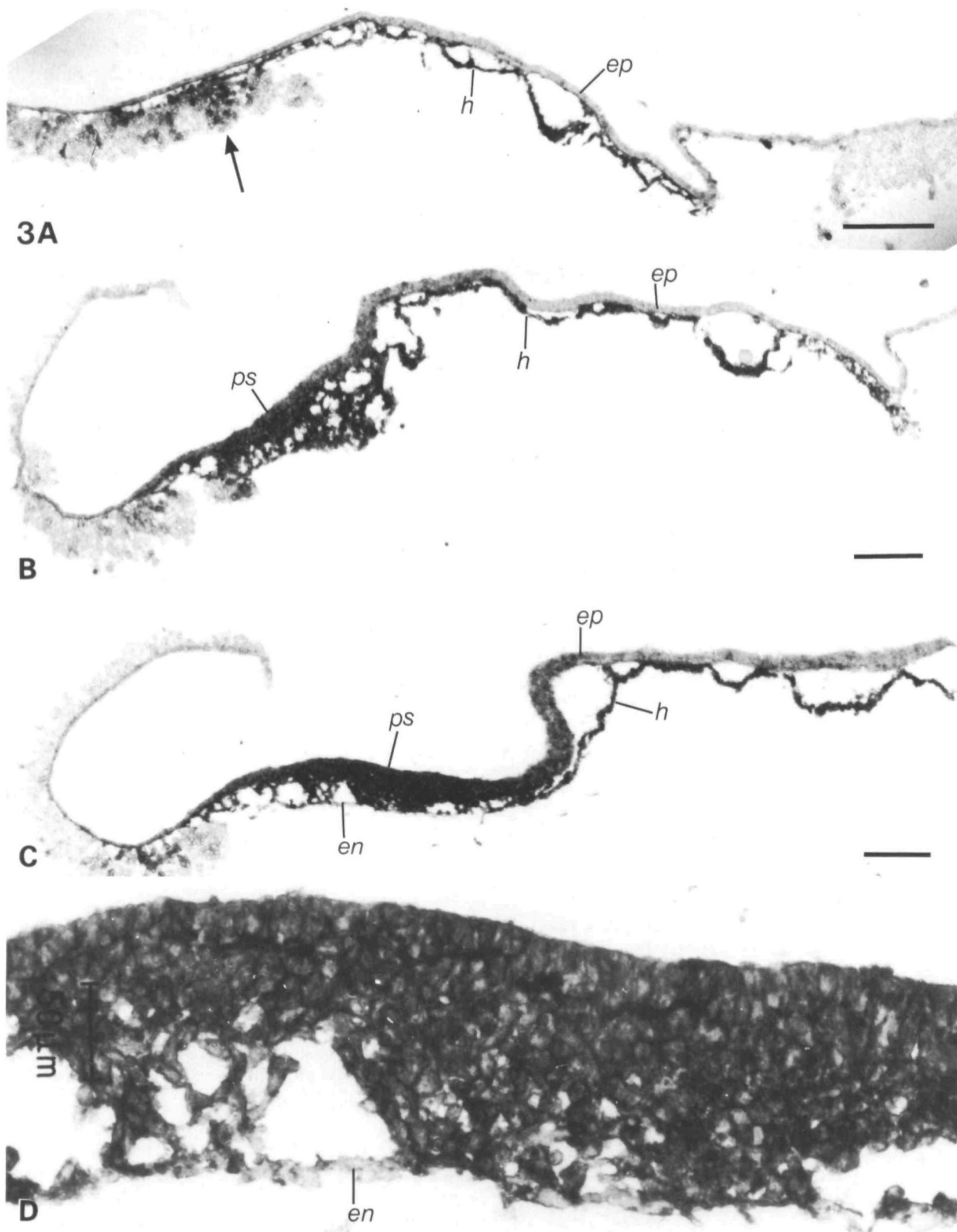
Fig. 2. Stages in the formation of the hypoblast as revealed by immunofluorescence. (A) Stage XI embryo. The main areas of staining in the hypoblast correspond to a single bright spot in each cell. Some of the overlying epiblast cells also stain with the antibody. (B) Phase-contrast photograph of the same area shown in A. (C) Stage XI embryo. The same pattern as in A can be seen, but at higher magnification a cell that appears to be ingressing from the epiblast to the hypoblast is HNK-1 positive and contains the single bright spot of fluorescence. Compare with the phase-contrast pair (D): the positive cell of the epiblast is associated with a depression in the apical surface of this tissue, consistent with this being a cell in the process of ingressing. (E-H) Stages XIII-XIV. By st. XIII (E,F) cells of the forming hypoblast sheet are strongly HNK-1 positive (rostral to the left of the photograph). When, at st. XIV, hypoblast formation is complete, all of its cells are strongly positive (G,H). Bars: B,F,H, 50 μ m; D, 20 μ m. *ep*, epiblast; *h*, hypoblast.

comparable to those found in the epiblast. Very faint bands are found at 105 and 41K, which may represent lower titres of the bands of equivalent molecular weight in the epiblast sample. A heavily staining band is found at 68K, which runs as a smear between 55 and 78K. Under reducing conditions, a band of 68K is

detected. A smear is seen between 60 and 46K, and a further minor band at 38K.

Hypoblast

Like the primitive streak, the hypoblast contains a



heavily glycosylated band at 68K which smears between 55 and 78K. A minor band is found at 41K, as is a very faint band at 105K. Under reducing conditions, a band of 68K is detected alone. A smear is seen between 60 and 46K, and a further minor band at 38K. This is similar to the pattern found in the primitive streak, but of less intensity. In addition to the above pattern, the hypoblast also contains a very faint band at 140K, which appears to be unique to this tissue.

Western blots

In order to test whether the pattern of expression of the HNK-1 epitope was a result of the presence of molecules of the HNK-1/L2 family, antibodies to the various members of the family and others were tested against the HNK-1-related proteins from each tissue, by Western blot analysis. None of the antibodies tested (N-CAM, G4, JG22/integrin, tenascin, J1) identified any of the HNK-1-related proteins under reducing or nonreducing conditions.

Discussion

The results presented in this paper show that tissues involved in the process of mesodermal induction in the chick embryo are identified by a monoclonal antibody, HNK-1. The antibody detects the cells of the hypoblast during its formation as well as later in development, a few cells in the posterior germ wall margin, the mesodermal cells of the primitive streak during its formation and some cells in the epiblast prior to primitive streak formation. After streak formation, the epiblast displays an anteroposterior gradient of HNK-1 expression. At the end of gastru-

lation, the primitive streak region loses its positivity. HNK-1 expression is next seen in the cells of the forming notochord and in the mesenchyme of the head fold.

Affinity-purified samples from st. XIV epiblast, st. 2-3 primitive streak and st. 2 hypoblast have characteristic patterns of HNK-1-related proteins after SDS-PAGE and silver staining: while all three tissues share some proteins, some are unique to each. In particular, under nonreducing conditions, a 32K band unique to the primitive streak and a very large (more than 300K) complex unique to the epiblast are very prominent. An investigation of the exact timing of expression of the 32K primitive streak specific glycoprotein may give a better understanding of the process of gastrulation. The nature of this glycoprotein and the possibility that it is present in other sites within the chick embryo are currently under investigation.

The HNK-1 epitope in relation to cell adhesion

The HNK-1 epitope is a complex, sulphated sugar moiety which is known to be promiscuous in its relation to the carrier proteins on which it is expressed. In the last few years, the epitope has been shown to be expressed on several cell surface glycoproteins apparently involved in adhesive interactions between neurones, glial cells and their matrices (Kruse *et al.* 1984, 1985; Grumet *et al.* 1985; Fushiki & Schachner, 1986; Cole & Schachner, 1987; Pesheva *et al.* 1987; Rathjen *et al.* 1987; Hoffman & Edelman, 1988). The carrier molecules have been classified together into a family owing to the fact that they all have the capacity to express the HNK-1 epitope. The HNK-1 epitope is recognized by another monoclonal antibody, designated L2, the specificity of which appears to be similar (Kruse *et al.* 1984). Among the molecules that express the HNK-1/L2 epitope are the major Ca^{2+} -independent cell-cell adhesion molecules, the neural cell adhesion molecules (N-CAMs) (Cole & Schachner, 1987) and the L1 group of antigens (Rathjen & Schachner, 1984; Keilhauer *et al.* 1985). Other molecules proposed to be members of the HNK-1/L2 family include the myelin-associated glycoprotein (MAG) (Kruse *et al.* 1984), integrin (Pesheva *et al.* 1987) and the glycoproteins J1 (Kruse *et al.* 1985) and F11 (Rathjen *et al.* 1987).

The HNK-1/L2 epitope is suggested to be important in the developing nervous system since it appears on macromolecules that are thought to be functionally important. The carbohydrate may modulate the activity of the cell adhesion proteins to which it is attached, since only some molecules of a particular molecular species carry the epitope (Kruse *et al.* 1984). Furthermore, Künemund *et al.* (1988) have shown that the HNK-1/L2 epitope itself can block

Fig. 3. Stages in the formation of the primitive streak, as seen by immunoperoxidase with HNK-1. All are sagittal sections through the midline of the embryo. (A) Stage XIV. The hypoblast (*h*) is positive, as are some cells at the caudal (left) margin of the germ wall (large arrow). Note the absence of these cells from the rostral (right) margin. The positive cells of the caudal margin appear to be continuous with the hypoblast. The basal lamina under the epiblast (*ep*) is also stained. (B) Stage 2. The primitive streak (*ps*) has now appeared and all of its cells appear to be positive. In addition, a gradient of HNK-1 staining is seen in the epiblast, increasing from rostral (right) to caudal (left). (C) Stage 3. The primitive streak is now packed with mesoderm cells which are strongly positive. The caudal germ wall margin still contains positive cells, and the epiblast still displays a gradient, which has now become more apparent. The cells of the lower layer beneath the streak (endoderm, *en*) are HNK-1 negative. This can be seen in more detail in the higher magnification photograph of the same section in D. Bars: A, 400 μm ; B,C, 200 μm .

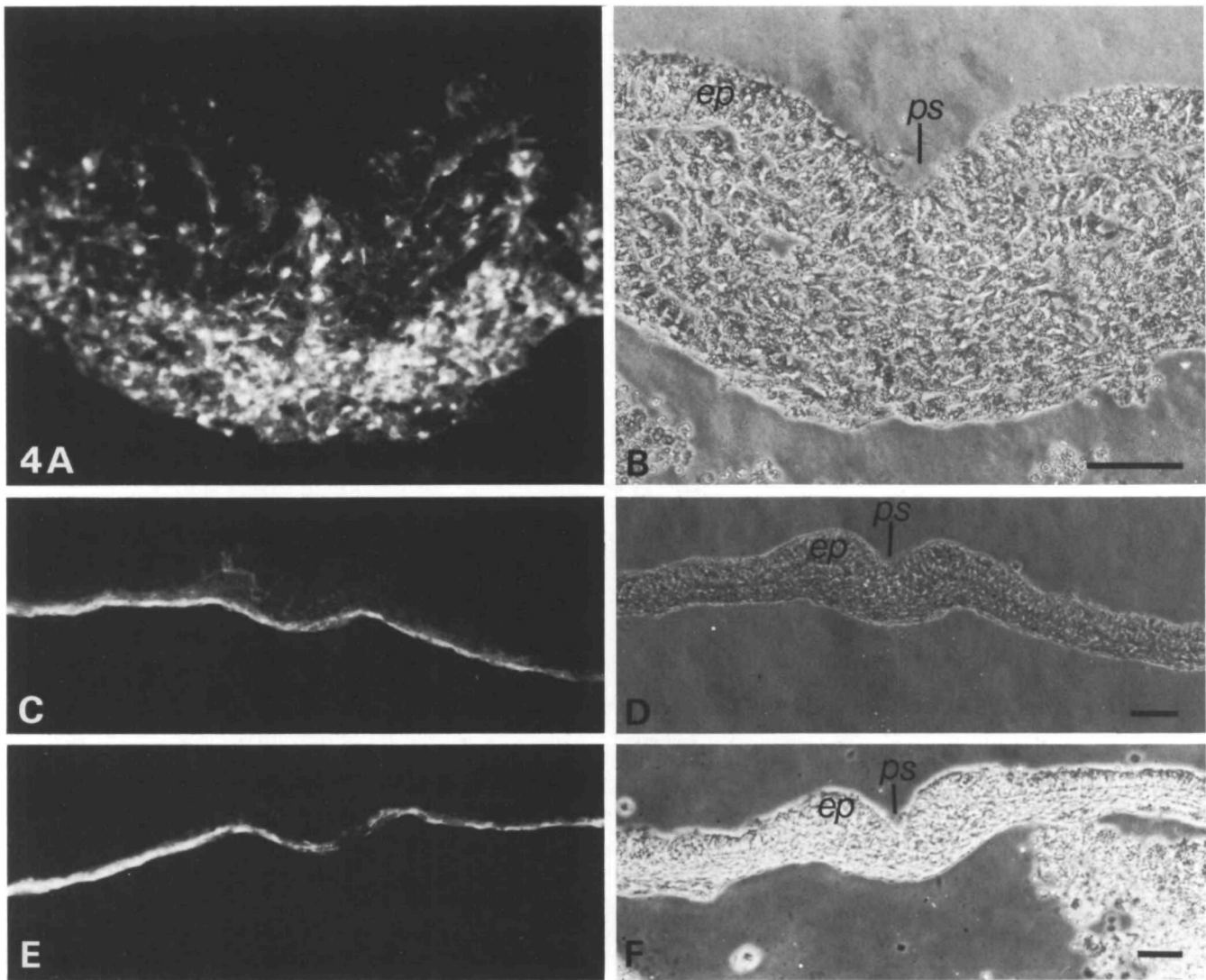


Fig. 4. Transverse sections through the primitive streak at stages 3⁺–4. (A) The cells of the mesoderm are strongly positive. Cells in the centre of the groove of the primitive streak and some more lateral cells in the epiblast are also positive. (B) Phase-contrast pair of A. (C,D) Stage 4⁻ (almost definitive streak stage). Positivity is now restricted to the most ventral portion of the mesoderm. The definitive endoderm now forms the lower layer and is HNK-1 negative. It can be seen in the phase photograph (D) as a very thin single layer of flattened cells. (E,F) Stage 4⁺ (late definitive streak stage). Positivity in the mesoderm is restricted to an even more confined ventral portion. All positivity has now disappeared from the epiblast. Bars: B, 50 μ m; D,F, 100 μ m. *ep*, epiblast; *ps*, groove of primitive streak.

cell–substrate adhesion. We found that a variety of mono- and polyclonal antibodies to the main known members of the HNK-1/L2 family do not react with the affinity-purified HNK-1 antigens present during chick gastrulation. Moreover, the molecular weights of the bands unique to each tissue do not correspond to those published for HNK-1-bearing molecules. We therefore suggest that the glycoproteins purified by affinity from primitive streak chick embryos may represent previously unidentified antigens of the HNK-1/L2 family.

Evidence is accumulating that the HNK-1/L2 family is not unique and that a system of carbohydrate epitopes affecting the function of adhesion molecules

may be widespread. Another family of neural cell adhesion molecules has been described recently, which is characterized by the L3 carbohydrate epitope (Kücherer *et al.* 1987). The L3 family includes some of, but not all, the adhesion molecules of the HNK-1/L2 family. This epitope resembles the HNK-1/L2 epitope in that the carbohydrate-carrying moieties are *N*-glycosidically linked and regulated independently of the protein backbone core. Furthermore, only a subpopulation of a particular adhesion molecule expresses the epitope. There appears to be partial overlap between the L3 and HNK-1/L2 families, L3 being expressed by the adhesion molecule on glia (AMOG), L1, MAG and by other as yet unident-

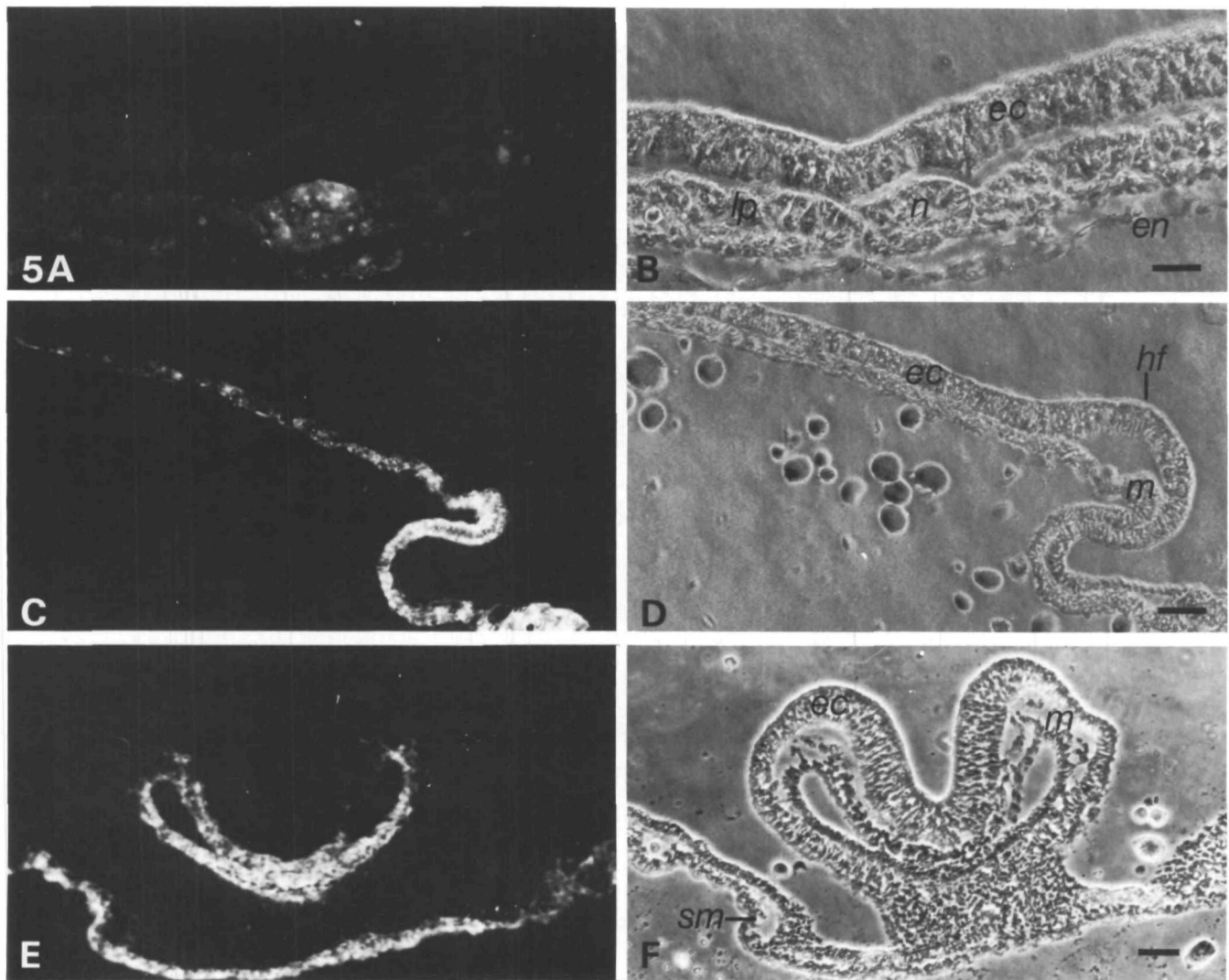


Fig. 5. Stages 5–6. (A,B) Transverse section through a st. 5 blastoderm at the level of the forming notochord. HNK-1 positivity is associated with the notochord (*n*), which shows the single spots of fluorescence similar to those seen earlier in the forming hypoblast. In addition, there are a few positive cells in the lateral plate mesoderm (*lp*). (C,D) Sagittal section through the forming head fold of a st. 6 embryo. The mesoderm of the head fold (*hf*) is positive, and the intensity of staining decreases towards the caudal (left) end. (E,F) Transverse section through the head fold of an embryo at st. 6. The mesoderm (*m*) of the head fold is positive, as is the splanchnopleural mesoderm (*sm*) ventral to it. Bars: B, 50 μ m; D,F, 100 μ m. *ec*, ectoderm (presumptive neural plate in A and B).

ified molecules of the nervous system, but not by N-CAM or J1.

The evidence surrounding these families of glycoproteins has led to the proposal that neural cell adhesion molecules are 'presenters' of functionally important carbohydrate structures (Kruse *et al.* 1984). With the large number of combinations possible, the repertoire of carbohydrate structures available for presentation is considerable, even with a small number of protein molecules eligible as presenters, and a small number of sugar epitopes to be presented. It has been stated (Lipinski *et al.* 1983) that the HNK-1 epitope is a marker of cells of neuroepithelial origin. Our results indicate that such

a system is not exclusive to the development of the nervous system and that this epitope is expressed during the formation of the embryonic axis.

The existence of a carbohydrate-based system that regulates protein function may help to explain why previous attempts to find regional protein differences in the early chick embryo have largely proved fruitless. It is possible that many of the differences between tissues appearing early in development are due to differences in carbohydrates rather than in the proteins expressed. This idea has formed the basis of an interesting model, which proposed that the structure of glycoconjugates at cell surfaces could form the basis for an 'epigenetic coding' system to specify

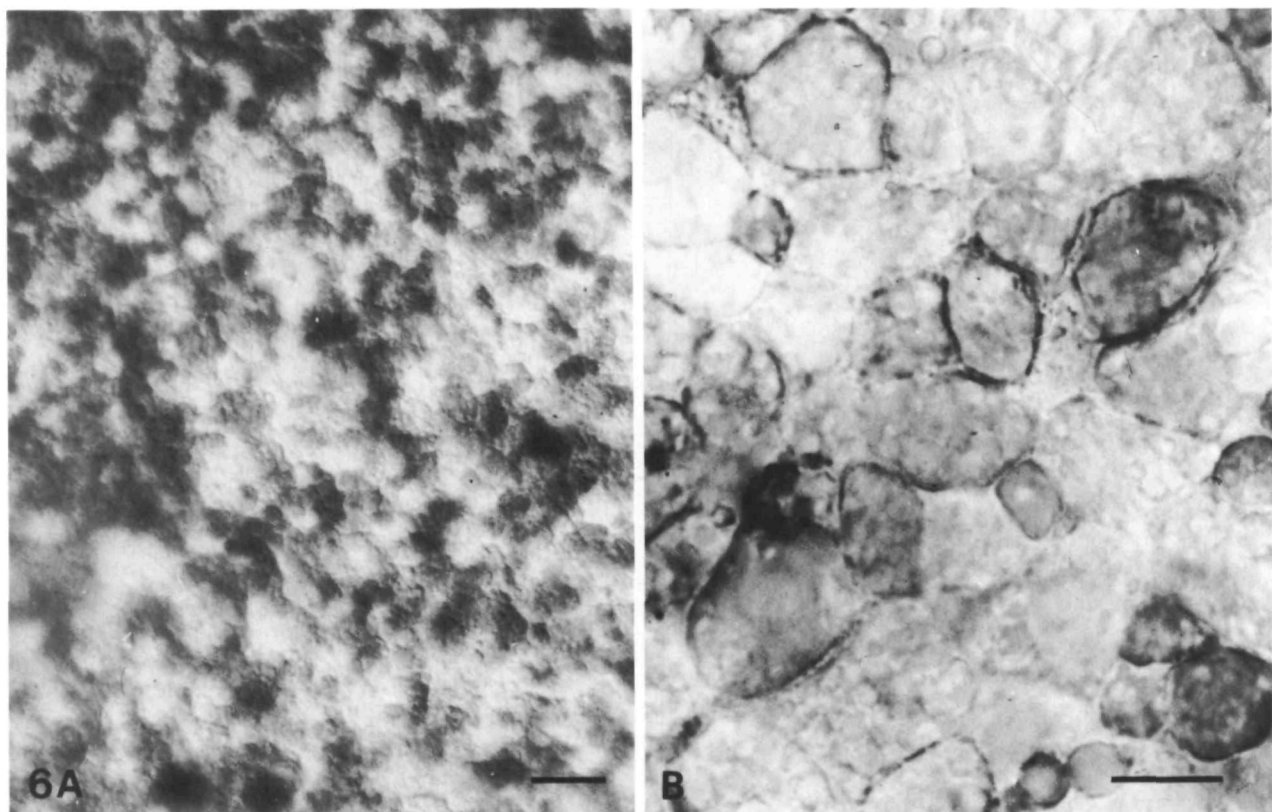


Fig. 6. Whole-mount immunoperoxidase staining patterns of the dorsal aspect of the epiblast at st. XII. Even at low magnification (A) it can be seen that the epiblast contains a mixture of HNK-1-positive and -negative cells. This is more apparent at high magnification (B). Note that although the size of the positive cells varies considerably, this variation appears to be no different from the distribution of sizes of negative cells. Bars, A, 15 μ m; B, 5 μ m.

Table 1. Summary of polypeptides separated by SDS-PAGE under nonreducing conditions from samples of the same tissues as shown in Fig. 7. The figures represent the relative molecular masses ($\times 10^{-3}$) of the polypeptides; large figures denote major components, while small figures show minor components. The bold numbers highlight those components unique to each tissue. Figures in brackets designate multiple bands obscured by a smear

Epiblast (stage XIV)	Hypoblast (stage 2)	Primitive streak (stage 2-3)
300+		
105	140	
78	105	105
71		
68	68 (55-78)	68 (55-78)
55		
41	41	41
		32

positional information (Slack, 1980). In support of this, Slack *et al.* (1985) have shown that several lectins reveal characteristic patterns during mesodermal and neural induction in *Xenopus* and axolotl embryos. In the chick, Thorpe *et al.* (1986) have recently shown that antibodies directed against specific sugar moieties reveal tissue-specific patterns during development, while Cook *et al.* (1979) and Zalik *et al.* (1987) have demonstrated the existence and studied the tissue distribution of carbohydrate-binding lectins.

Our results and those of others suggest that HNK-1 expression plays a role in early developmental processes: during gastrulation, expression is greatest in regions undergoing the most obvious morphogenetic changes. At the end of gastrulation, embryonic regions no longer display the epitope: the epiblast and mesoderm down-regulate its expression, while the hypoblast has been displaced away from the embryonic region. Evidence from *in vitro* studies of neuronal cells indicate that the HNK-1 epitope itself is a functionally important epitope involved in cell-substrate and cell-cell interactions (Künemund *et al.*

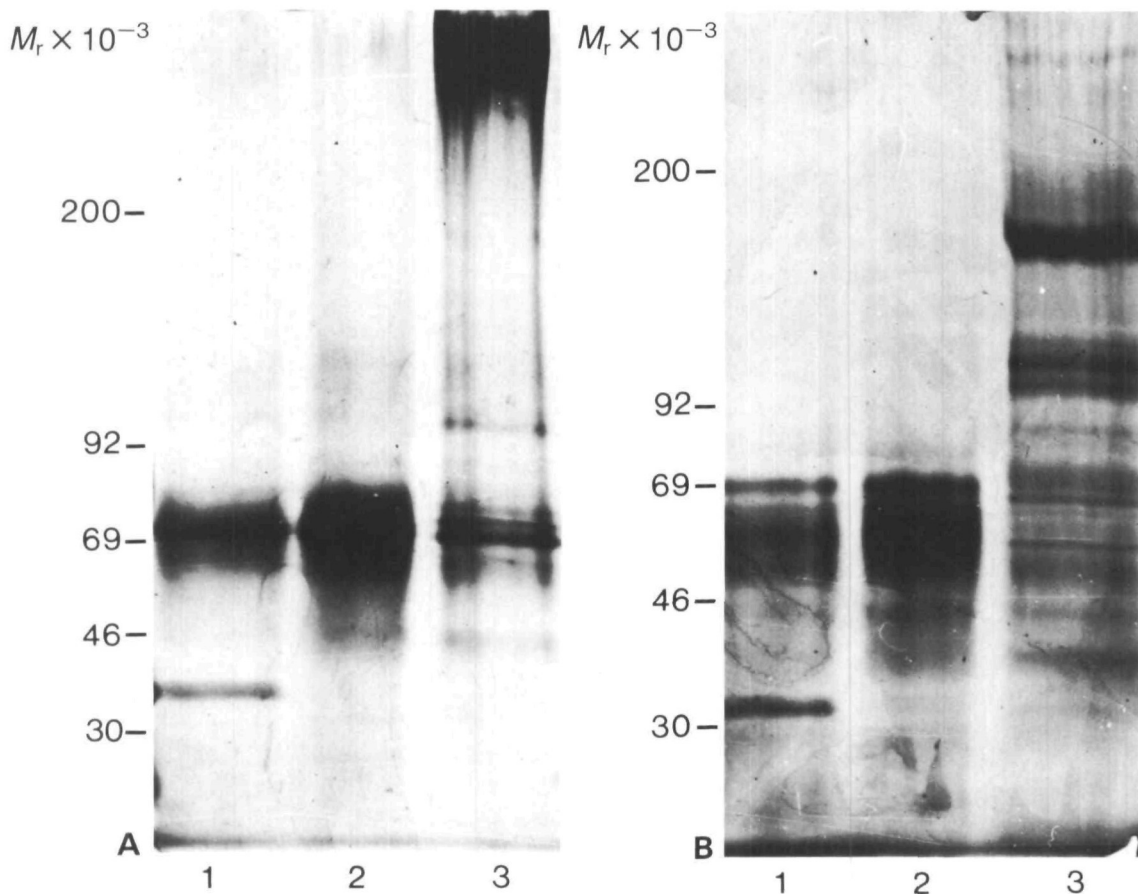


Fig. 7. Protein separation of affinity-purified samples of primitive streak (lane 1), hypoblast (lane 2) and epiblast (lane 3) under nonreducing (A) and reducing (B) conditions. Silver-stained gel.

1988). It is therefore difficult to imagine that HNK-1 expression during gastrulation is incidental.

The HNK-1 epitope and hypoblast formation

Our results show that the hypoblast expresses the HNK-1 epitope from the earliest stage of its formation, and that it continues to express it even after its localization has become restricted to the anterior germinal crescent region. The origin of the hypoblast appears to be dual, receiving contributions from the epiblast by ingression at many different sites and from the caudal margin of the germ wall (*area opaca* endoderm) (see, for example, Stern & Ireland, 1981; Vakaet, 1984; Stern & Canning, 1988). Vakaet (1984), among others, has advanced the view that the islands seen in young stages constitute a primitive, or *primary*, hypoblast, and that the *secondary* hypoblast that completes the primitive lower layer is derived from the germ wall margin, particularly at the caudal margin of the blastodisc.

Our present results are consistent with this view. We find cells expressing HNK-1 immunoreactivity associated with the lower surface of the epiblast at stage X, during which period polyingression is said to

be taking place, as well as in the posterior germ wall margin at later stages, when this region is said to contribute cells to the lower layer (Stern & Ireland, 1981). Azar & Eyal-Giladi (1979) and Mitrani *et al.* (1983) have suggested that it is this secondary hypoblast that is responsible for inducing the primitive streak.

The HNK-1 epitope in relation to induction

Craniocaudal rotation of the hypoblast by 180° prior to primitive streak formation affects the orientation of the embryonic axis so that it follows that of the hypoblast (Waddington, 1930, 1933; Azar & Eyal-Giladi, 1981). These experiments have been taken as evidence in favour of the notion that the hypoblast exerts an inductive influence on the overlying epiblast. In addition, transfilter experiments have provided evidence for a weak inductive signal between the hypoblast and the epiblast, although formation of a complete primitive streak requires direct contact (Eyal-Giladi & Wolk, 1970).

Since both the hypoblast and the primitive streak express HNK-1, it is not inconceivable that the interaction between the primitive streak and the

hypoblast may be associated with the expression of the HNK-1 epitope. The basal lamina is HNK-1 positive as the epiblast reaches the stage at which it becomes competent to form a primitive streak. At this stage, HNK-1 positivity of the basal lamina involves a large molecular weight complex, which is heavily glycosylated. Vanroelen & Vakaet (unpublished results) have demonstrated that a sugar transport system exists between the hypoblast and the epiblast basal lamina, using [^3H]glucosamine-labelled grafts of hypoblast tissue to unlabelled epiblasts. Since it is uncertain that protein synthesis is necessary for induction to take place (see Gurdon, 1987), it may be that the hypoblast is imparting sugars to the epiblast and that these carbohydrates in some way modify particular adhesion molecules, initiating the epithelium-to-mesenchyme conversion. The intracellular spot of HNK-1 staining seen in the hypoblast (see Fig. 2A,C) could correspond to the Golgi apparatus, which could therefore be the intracellular site of glycosylation of the HNK-1 bearing molecules. If this is true, the idea that the hypoblast confers HNK-1 positivity to the epiblast cells receives support from the finding that HNK-1-positive epiblast cells do not display this intracellular staining.

It is difficult to correlate the mosaicism prior to primitive streak formation with the rostrocaudal gradient in the midline of the epiblast during streak elongation: just prior to primitive streak formation, the epiblast is a mixture of cells that are either positive or negative, while immediately after streak formation (1–2 h later) the gradient seen in the epiblast reflects an orderly progression of quantitative expression of the epitope, which does not seem to be due to differences in the proportion of positive and negative cells. We are not yet in a position to speculate on the significance of the gradient.

The appearance of the mosaic of HNK-1-positive and -negative cells in the epiblast prior to gastrulation is somewhat puzzling. It is possible that the HNK-1-positive cells are a distinct population with the potential to give rise to streak mesoderm. If this notion is correct, the competence of the epiblast might arise by a stochastic mechanism, with the initiation of the streak being governed by a local selection of competent cells from the epiblast. A second possibility is that the HNK-1-positive cells of the mosaic might be the precursors of the positive cells of the primitive streak. If this is correct, the hypoblast might act by taking part in sorting out positive from negative cells in the epiblast. At first sight, both views appear to conflict with the idea that induction of the primitive streak is an *instructive* event. However, these are not the only possible explanations of the pattern seen.

At the end of gastrulation, the mesoderm adjacent

to the primitive streak ceases to express HNK-1 positivity. Soon afterwards, however, the notochord is the next tissue to become HNK-1-positive. Since this tissue is known to be capable of *neural* induction, it is tempting to speculate that HNK-1/L2 or related sugars might be a general feature of inductive interactions.

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