Development 118, 553-562 (1993)
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The chicken *CdxA* homeobox gene and axial positioning during gastrulation

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SUMMARY

The chicken homeobox containing gene, *CdxA* (formerly *CHox-cad*), was previously shown to be expressed during gastrulation. Localization of *CdxA* transcripts by in situ hybridization to tissue sections revealed that, during gastrulation, expression of this gene exhibits a posterior localization along the primitive streak. The transcripts are localized to epiblast cells in the vicinity of the primitive streak, to cells of the primitive streak itself and in the definitive endoderm as it replaces the hypoblast.

In order to study in greater detail the pattern of expression of the *CdxA* gene during gastrulation, we expressed the full-length CdxA protein as a fusion protein in *E. coli* and generated monoclonal antibodies against it. Chicken embryos at different stages of gastrulation were processed for whole-mount immunohistochemical localization of the protein using anti-CdxA antibodies. Once the pattern of expression in the whole embryo was determined, the same embryos were sectioned to determine the identity of the cells expressing the CdxA protein. Detailed analysis of the CdxA protein in embryos, from the onset of primitive streak formation to the beginning of the tail bud stage (stages 2 to 10), has shown different patterns of expression during primitive streak elongation and regression. The CdxA protein is initially detected at the posterior marginal zone and the expression moves rostrally into the primitive streak during mid-streak stages. As the primitive streak elongates, the CdxA stripe of expression moves anteriorly. By definitive streak stages, the CdxA stripe of expression delineates a position along the anterior-posterior axis in the primitive streak. *CdxA*, like its *Drosophila* homologue *cad*, is expressed during gastrulation in a stripe localized to the posterior region of the embryo. These observations suggest that *CdxA* as a homeobox gene may be part of a regulatory network coupled to axial determination during gastrulation in the early chick embryo.

Key words: gastrulation, homeobox genes, endoderm, axial specification, chicken embryos

INTRODUCTION

Gastrulation is the process by which the bilayered embryo composed of the epiblast, the upper layer, and the hypoblast underneath it, generates the three germ layers, the ectoderm, mesoderm and endoderm. A structural manifestation of the process of gastrulation in birds and mammals is the primitive streak (PS), and it is also one of the earliest structures marking the embryonic craniocaudal axis. PS formation starts at the posterior end of the embryo with the de-epithelialisation of prospective mesendodermal cells from the epiblast, and their ingression towards the midline (Bellairs, 1986; Stern, 1991; Nicolet, 1971). The ingression of cells from the epiblast and their accumulation to form the PS is one of the first events during gastrulation in the chicken embryo. Next comes the movement of cells in the PS and the epiblast, first in a ventral direction along the streak and later away from it, mainly in a lateral direction to form the mesoderm and definitive endoderm (Bellairs, 1986; Stern, 1991). During the reorganization of the epiblast, the formation of the streak and the mesodermal and endodermal layers, the PS exhibits changes in length and shape (Hamburger and Hamilton, 1951; H&H). During early stages of gastrulation, the PS elongates from the posterior towards the anterior end of the embryo, and then, at later stages, it regresses until it becomes part of the tail bud (Schoenwolf, 1979). All these events taken together support the notion of extensive cell movements during gastrulation. The fate

of cells migrating through the PS changes with the age of the embryo. During PS elongation ('progression'; stages 3-4$^+$; H&H), cells ingressing through the cranial part of the streak are destined to become mostly endoderm (Nicolet, 1967, 1970; Stern, 1991), while cells migrating through its posterior regions will become mostly extraembryonic mesoderm (Nicolet, 1967, 1970). Prospective mesodermal cells begin ingressing during late stages of PS elongation (about stage 4; H&H) and continue doing so during streak regression (Nicolet, 1967, 1970; Schoenwolf, 1991; Stern, 1991).

The mode of cell movements during gastrulation can be divided chronologically into (i) cells joining the PS during elongation, 'ingression', (ii) cell movement ventrally along the PS, (iii) cells leaving the streak, 'migration' and (iv) cell movements during PS regression. Delamination of cells from the epiblast to the space between their original cell layer and the hypoblast, and their accumulation at the posterior end of the embryo, initiate the formation of the PS (Bellairs, 1986; Stern, 1991). The elongation of the PS takes place apparently by a process similar to 'convergent extension' (Keller et al., 1991; Stern, 1991). This process has been described as epiblast cells joining the PS close to its anterior end and then moving either anteriorly or posteriorly, resulting in the elongation of the streak (Nicolet, 1971; Bellairs, 1986; Malan, 1953). The onset of migration is marked by the cells that start to leave the PS to form the mesodermal and endodermal layers. (Nicolet, 1971; Bellairs, 1986).

A number of treatments can result in the formation of extranumerary axes or inhibit axis formation. In *Xenopus* embryos, the injection of RNAs derived from *activin* or *Wnt* genes results in the formation of extra axes (Thomsen et al., 1990; Nusse and Varmus, 1992). In chicken embryos, the removal of the marginal zone, the area opaca and the hypoblast results in the inhibition of axial structures and normal development (Azar and Eyal-Giladi, 1979). Activin treatment of chicken embryos can generate ectopic axial structures (Ziv et al., 1992), and also generate axial structures even in embryos manipulated so as to prevent axis formation (Mitrani et al., 1990).

Few molecular markers are available to help us understand the regulation and the interactions taking place during gastrulation. As regards to the available molecular markers, it is of interest to note their rostrocaudal distribution during gastrulation, and their distribution along the three germ layers. The murine *goosecoid* gene is expressed during PS elongation at the anterior end of the streak and later becomes restricted to the anterior most mesoderm (Blum et al., 1992). The *Xenopus goosecoid* is expressed in the dorsal lip of the blastopore, Spemann's organizer (Cho et al., 1991). Spemann's organizer has been suggested to be a structure similar in function to the anterior end of the PS in mice or chickens, the primitive node or Hensen's node (Cho et al., 1991; Kintner and Dodd, 1991). There are other genes known to be expressed in the blastopore lip in *Xenopus*. *Xlim-1* is expressed both in the dorsal lip and the dorsal mesoderm in the region of Spemann's organizer (Taira et al., 1992). *Fork head* is expressed in the dorsal lip and in the chordal mesoderm (Dirksen and Jamrich, 1992). Finally, *noggin* is expressed in Spemann's organizer, notochord and prechordal mesoderm (Smith and Harland, 1992). Some of these genes are putative transcription factors, while others probably code for secreted proteins. Additional genes have been described that are expressed in specific germ layers during gastrulation or other regions of the PS. The *Mix.1* gene from *Xenopus* is expressed in the prospective endoderm (Rosa, 1989). The chicken *Hox 2.9* gene is expressed all along the PS and in the ectoderm and mesoderm (Sundin et al., 1990). The murine *Hox 1.5* gene is expressed in the same germ layers as *Hox 2.9* but it is restricted to posterior regions of the embryo (Gaunt, 1987). Posterior expression has also been described for the murine *Evx-1* gene which is expressed in all three germ layers but it exhibits a posterior gradient (Dush and Martin, 1992). In addition to putative regulatory proteins like the above mentioned homeobox genes, there are a number of growth factors that exhibit spatially localized patterns of expression in the mouse embryo. One such gene is the *Fgf-4* gene whose transcripts localize to the rostral two thirds of the PS and to the epiblast cells joining the streak (Niswander and Martin, 1992).

During gastrulation, one of the homeobox genes that exhibits an intriguing spatial localization is the chicken *CdxA* gene (formerly *CHox-cad*, Frumkin et al., 1991). Analysis by in situ hybridization revealed that its transcripts are localized to cells in the epiblast joining the PS, cells in the streak itself and mesodermal cells leaving the PS. In addition, *CdxA* transcripts were detected in the definitive endodermal cells. This cell type distribution also exhibited a rostrocaudal restriction, the *CdxA* transcripts showed a predominantly posterior localization whose levels decreased in a cranial direction.

In order to determine in greater detail the pattern of expression of *CdxA* during gastrulation, we have developed monoclonal antibodies against its protein product (CdxA). Whole-mount immunohistochemistry followed by histological sectioning and analysis were utilized to determine the position and the changes of the *CdxA* pattern of expression during gastrulation. Expression of CdxA begins shortly after the onset of PS formation and can be observed as a wave of expression that moves rostrally along the PS. During definitive streak stages, the CdxA protein is localized in a stripe about one third from the posterior end and its levels decrease rostrally and caudally. The cellular distribution observed supports our suggestion that the *CdxA* gene is expressed in definitive endodermal cells as this layer is established.

MATERIALS AND METHODS

Embryonic material

Fertilized eggs were purchased from local farms. Eggs were incubated at 37.7°C and rotated every hour. Embryos were incubated for different time periods until they reached the desired developmental stage. The embryos were dissected out in ice-cold phosphate-buffered saline (PBS) and staged according to Hamburger and Hamilton (1951). Fixation was performed in 20% dimethyl sulphoxide (DMSO) in methanol, overnight at 4°C (Dent et al., 1989). Endogenous peroxidase activity was destroyed by transferring the embryos to 5% hydrogen peroxide in fixative and incubated 4-5 hours at room temperature. The embryos were transferred to 100% methanol and stored at −20°C.

Preparation of monoclonal antibodies

The CdxA protein was prepared in *E. coli* as a fusion protein with glutathione-S transferase. The cDNA clone for *CdxA*, C33 (Frumkin et al., 1991), was digested with *Afl*III which cuts 2 bp upstream from the codon for the suggested initiator methionine. This same enzyme cuts the cDNA again about 800 bp downstream from the stop codon of the homeodomain containing open reading frame. The 1545 bp long *CdxA* fragment was subcloned into the *Sma*I site of the pGEX-2T vector to produce a fusion protein with the glutathione-S-transferase (GST) coded by it (Smith and Johnson, 1988). The fusion protein was induced and prepared according to Smith and Johnson (1988). Affinity purification of the fusion protein was performed on glutathione-agarose beads (Sigma). Balb/c mice were immunized with the fusion protein still bound to the beads. After the second boost, hybridomas were prepared as described (Harlow and Lane, 1988). Spleen cells were fused to the P3X63-Ag8.653 myeloma cell line. Hybridomas were initially screened by spotting the fusion protein. Positive clones were re-screened by reacting the antibody against pairs of spots one containing the fusion protein and the other only GST. Clones positive for the fusion protein and negative for GST were tested by protein blot analysis.

Whole-mount immunohistochemistry

Whole-mount immunohistochemistry was performed according to Dent et al. (1989) and Davis et al. (1991) with several modifications. The embryos stored at −20°C were rehydrated to PBS and incubated for 30 minutes in blocking solution containing 0.5% Triton X-100 and 20% skim milk in PBS (PBSMT). The first antibody, 6A4αCdxA, was diluted in PBSMT and the embryos were incubated overnight at 4°C. The 6A4αCdxA antibody was diluted 1:1 if tissue culture supernatant was used, and 1:150 if ascites fluid was utilized. After incubation, the embryos were washed 7 times with PBSMT for at least 30 minutes each wash. The second antibody, donkey anti-mouse IgG-Fab fragment coupled to horseradish peroxidase (Jackson Immunoresearch) was diluted 1:1500 in PBSMT. The embryos were incubated with the second antibody overnight at 4°C, and washed as before. Staining was performed with 3,3′-diaminobenzidine (tetrahydrochloride) as described by Davis et al. (1991) either in the presence or absence of 0.5% $NiCl_2$ resulting in black or brown staining, respectively. After staining, the embryos were cleared in 100% glycerol.

Histological analysis

After clearing, the stained embryos were photographed and then processed for histological analysis. The embryos were embedded in paraffin and 5-8 μm serial sections were collected on TESPA (3-amino-propyltriethoxysilane)-treated glass slides. The slides were treated with xylene to remove the paraffin. The sections were counterstained with Fast green and mounted with Entellan (Merck).

Protein blot analysis

Embryonic or bacterially produced proteins were electrophoresed on SDS-polyacrylamide gels and semi-dry blotted onto nitrocellulose. Detection of the CdxA protein was performed by blocking the membrane in 0.1% Tween-20, 5% skimmed dry milk (powder) in PBS. The first antibody, 6A4αCdxA from ascites was diluted 1:5000 in PBS, 0.1% Tween-20 and 1% skimmed dry milk and was incubated with the blot overnight at 4°C. After three washes for 10 minutes each in PBS containing 0.1% Tween-20, the second antibody, donkey anti-mouse IgG-Fab fragment coupled to horseradish peroxidase (Jackson Immunoresearch), which was diluted 1:50,000 in PBS-Tween-1% milk, was added and incubated for 2 hours at room temperature. After washing as before, the peroxidase reaction was performed with the ECL kit (Amersham) as recommended by the manufacturer.

RESULTS

Antibodies against the CdxA protein

In order to study in detail the *CdxA* pattern of expression, antibodies directed against its protein product were raised. The CdxA protein was produced in bacteria as a fusion protein with glutathione-S-transferase (GST; Smith and Johnson, 1988). The GST-CdxA fusion protein after its purification by affinity chromatography on agarose-glutathione beads, was utilized to immunize mice to generate monoclonal antibodies against CdxA. Hybridoma clones were initially screened by testing in parallel spots containing the GST-CdxA fusion protein or only the vector-produced GST protein. Clones positive for GST-CdxA and negative for GST were tested against both proteins by protein blot analysis. Specificity of the monoclonal antibodies was initially tested against a protein produced in an independent procaryotic expression system, pKK223-3 (Brosius et al., 1984). In this expression system, we produced non-fusion CdxA protein translated from its putative initiator methionine. The monoclonal antibodies were tested against crude bacterial protein extracts containing either the *CdxA* insert in the plasmid or the original plasmid with no insert. Fig. 1A shows the results of the protein blot analysis using the 6A4αCdxA monoclonal antibody. The results demonstrated that this antibody recognizes only the CdxA protein produced in *E. coli* but no other proteins from the bacteria as seen from the control lane. In order to determine whether the 6A4αCdxA monoclonal antibody recognizes chicken embryonic proteins, protein extracts were prepared from chicken embryos and tested by protein blotting (Fig. 1B). Whole protein extract was prepared from stage 11 (H&H; 13 somites) chicken embryos which is an embryonic stage during which it has been shown the *CdxA* mRNA is present. Detection has to be performed at very high sensitivities due to the low abundance of the CdxA protein. As can be seen the 6A4αCdxA antibody mainly recognizes a protein of about 37×10^3 M_r (Fig. 1B). The calculated molecular weight of the CdxA protein is about 33×10^3 M_r therefore this is probably the CdxA protein. It was also of interest to determine the general localization of the epitope recognized by the 6A4αCdxA antibody. For this purpose, we generated a number of deletions of the GST-CdxA fusion vector keeping always the amino terminus of the CdxA protein joined to GST (Fig. 1C). One of the deletions GST-CdxA-18, was generated by digestion with *Pst*I leaving a portion of the CdxA protein from the amino terminus to 4 amino acids downstream from the homeodomain. The second deletion GST-CdxA-15, generated with *Sma*I extends from the amino terminus to 61 amino acids upstream to the homeodomain. As shown in Fig. 1D the 6A4αCdxA antibody recognizes all the deleted CdxA proteins and part of their degradation products, mapping the epitope to the region upstream from the homeodomain and the hexapeptide (Frumkin et al., 1991).

CdxA localization during primitive streak elongation

To study in detail the spatial and temporal pattern of expression of the CdxA protein, the 6A4αCdxA mono-

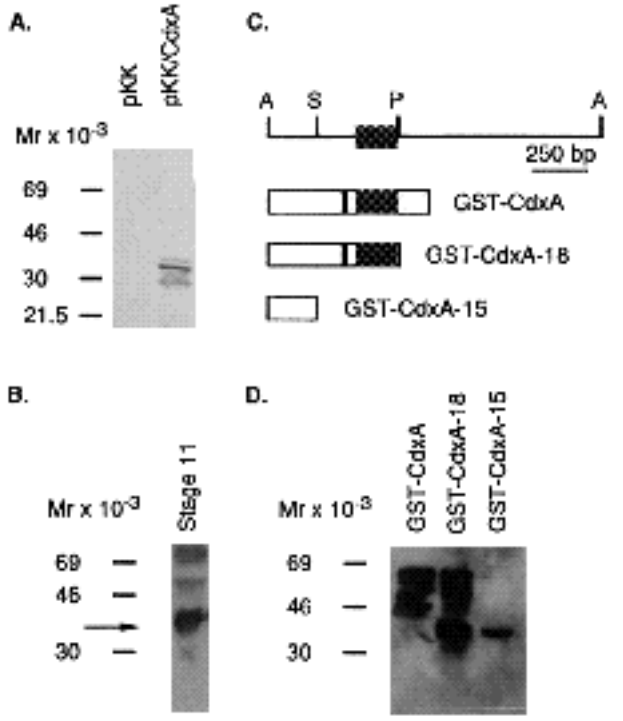

Fig. 1. Characterization of the 6A4αCdxA monoclonal antibody. (A) Detection of the CdxA protein prepared in the pKK system. The CdxA protein was prepared in a second expression system different from the one used to generate the monoclonal antibodies. Whole protein extracts from bacteria were utilized. Lane 1 contains a protein extract from cells carrying the pKK223-3 vector only, and lane 2 contains the CdxA producing construct. (B) Detection of the CdxA protein from embryonic extracts. Total protein extracts were prepared form embryos a stage 11 (13 somites) and processed for protein blotting. Detection of the CdxA protein was performed with the 6A4αCdxA antibody. (C) Diagram of the protein fragments obtained from the different deletions generated to determine the epitope recognized by 6A4αCdxA antibody. Two truncations of the CdxA protein were prepared; in both cases the region kept is the amino terminus of the protein. The homeodomain region is marked as a gray box and the hexapeptide as a black box. (D) Protein blot detection of the truncated CdxA proteins to map the position of the 6A4αCdxA epitope. Lane 1 contains a protein extract that produces the full-length GST-CdxA fusion protein, lane 2 contains the fusion protein produced from GST-CdxA-18, which results in a fusion protein that is truncated 4 amino acids downstream from the homeodomain. Lane 3 is the protein extract from GST-CdxA-15 where the fusion protein was truncated upstream from the homeodomain. In all three cases, the fusion proteins and their degradation products are detected.

clonal antibody was utilized for immunohistochemistry of whole-mount chicken embryos. Chicken embryos were incubated for periods up to 2 days and then processed for whole-mount immunohistochemistry. In all stages, the embryos stained as whole mounts were processed for histology, and serial sections were obtained to determine the identity of the cells in which the protein is expressed. The CdxA protein is first detectable in embryos at stage 3-3$^+$ (H&H), when the embryo is well into gastrulation, ingression is underway and the PS has already acquired its elongated form. At this developmental stage, the protein is distributed along the marginal zone, the junction between the area pellucida and the area opaca. Along the sides of the area pellucida, it shows an anterior boundary of expression that matches the rostral end of the PS (Fig. 2A). At the caudal end of the embryo, the CdxA protein is found in cells just rostral to the posterior marginal zone (Fig. 2A). The posterior marginal zone has been shown to determine the position of the PS and in some instances to be responsible for the formation of supernumerary axes in the embryo (Khaner and Eyal-Giladi, 1986). Cell type analysis of the expression pattern by serial sectioning showed that along the marginal zone, CdxA is expressed in the yolky germ wall cells (Fig. 3A). The expression extending rostrally from the marginal zone at the posterior end, localizes to cells contiguous with the epiblast (Stern, 1990). At stage 3$^+$, the localization of the CdxA protein along the lateral marginal zone described for the earlier embryos can still be observed. The main difference in the distribution of the CdxA protein is seen at the caudal end of the embryo. At this developmental stage, the CdxA protein can no longer be detected in the region just rostral to the posterior marginal zone. Interestingly, the posterior portion of the PS now exhibits CdxA staining (Fig. 2B). This shift in the spatial position of the posterior staining pattern suggests that the posterior labeling has moved rostrally into the PS. In the labeled portion of the PS, we can see staining in all cells of the streak itself (Fig. 3B). In the close vicinity of the PS, all cells in the epiblast and mesoderm are labeled. The lateral boundary of the CdxA-positive cells on either side of the PS extends about 7-10 cell diameters towards the side. The germ layer exhibiting the most extensive labeling along the mediolateral axis is the newly formed definitive endoderm, where all cells extending up the germ wall are labeled (Fig. 3B). Detailed analysis of the sectioned material revealed unlabeled regions of the PS rostral and caudal to the CdxA stripe of expression. Clear anterior and posterior boundaries along the PS further support our observation that the CdxA stripe has moved into the PS and even moved along it.

During stages 4 and 4$^+$, as the PS reaches its maximal length, the marginal zone staining disappears. As the marginal zone staining becomes undetectable, the CdxA stripe of expression continues to move forward along the PS (Fig. 2C, D). At the definitive streak stage, the CdxA stripe shows a clear peak along the posterior half of the PS (Fig. 2D). The levels of CdxA protein decrease gradually anteriorly and posteriorly. The bidirectional decrease along the stripe could result from fewer cells expressing the protein as they are located farther from the peak of expression. A second possibility is that all cells continue to express the CdxA protein but the levels of expression decrease. Detailed analysis of the serial sections of the embryo in Fig. 2D and others at the same developmental stage revealed that the bidirectional decrease in CdxA protein levels results from lowered protein levels in each cell and not from a decrease in the number of CdxA expressing cells (Fig. 3C).

CdxA expression during primitive streak regression

At about stage 5 (H&H), regression of the primitive streak begins. As the regression of the PS becomes evident, the spatial localization of the CdxA protein changes. The first

difference is that from a stripe-like pattern along the elongating PS, expression of the CdxA protein spreads to the whole length of the streak (Fig. 4). This expression extends from Hensen's node to the posterior end of the PS (Fig. 4). This expression pattern can be observed through the different stages of PS regression, from the onset of regression (Fig. 4A), up to stage 10 (10 somites) when the CdxA protein becomes undetectable (Figs 4D, 3F). As the PS becomes shorter, the CdxA protein continues to be expressed from Hensen's node to the posterior end of the streak (Fig. 4A-C). A second change in the distribution of the CdxA protein was detected at the cellular level when embryos during PS regression were stained and sectioned. Not only the rostrocaudal restriction of the CdxA protein along the PS is lost, but its cellular distribution has changed to an extent. During regression, CdxA is present in all cells of the PS. Expression also localizes to the epiblast and mesoderm cells in the vicinity of the PS but no longer in definitive endoderm cells (Fig. 3D, E). This lack of definitive endoderm labeling could be attributed to the fact that during regression very few cells that migrate through the PS become endoderm (Nicolet, 1967, 1970).

An interesting observation was that at stage 10 (H&H; 10 somites), the CdxA protein becomes undetectable. In an attempt to corroborate this observation by a different approach, we performed western analysis on crude protein extracts from embryos at stages 9 to 11 (H&H). The protein extracts were prepared from embryos staged according to the number of somites (Hamburger and Hamilton, 1951). Detection of the CdxA protein was performed with the 6A4αCdxA monoclonal antibody followed by chemiluminiscence. Our results from this kind of approach revealed that the level of the CdxA protein does indeed decrease as the embryos approach the 10 somite stage (data not shown). These experiments were repeated either loading protein extracts from equal numbers of embryos or similar protein quantities. In all instances, we obtained similar results supporting the decrease in CdxA levels at stage 10. Although the two approaches, immunoblotting and immunohistochemistry, differ in their sensitivities both supported the notion of lower CdxA protein levels at stage 10. The formation of the tail bud begins at about stage 10 and it apparently requires a different organization of the posterior end of the embryo and the PS (Schoenwolf, 1979). Therefore, the formation of the tail bud could be a possible reason for the effect on the expression of the CdxA protein.

DISCUSSION

The *CdxA* stripe of expression

At the full streak stage in chicken embryos, the CdxA protein is expressed as a stripe intersecting the PS. The fact that this stripe has rostral and caudal boundaries within the PS was corroborated by analysis of serial sections of stained embryos. The anterior and posterior margins of this stripe are clearly discernible but expression decreases in both directions in a gradient fashion. The posterior margin of the stripe localizes about 1/3 of the PS length from the posterior end. The expression of CdxA as a stripe that traverses the embryonic anteroposterior axis is reminiscent of the patterns of expression of some genes in the *Drosophila* embryo. During embryogenesis in *Drosophila*, axial positions along the anteroposterior axis are determined by genes that are expressed as stripes that intersect this axis. The first group of genes expressed from the zygotic genome, the gap genes, are expressed as broad bands along the axis of the embryo and between them begin subdividing the embryo along its anteroposterior axis (Hülskamp and Tautz, 1991; St. Johnston and Nüsslein-Volhard, 1992). This similarity suggests that the stripe of *CdxA* expression represents a molecular marker of events leading to the rostrocaudal regionalization of the PS and subsequently, the embryo. This suggestion is further supported by experiments performed in *Xenopus* embryos where it was concluded that an anteroposterior pattern in the mesoderm is established during gastrulation (Gerhart et al., 1989; Slack and Tannahill, 1992). One of the questions raised by this pattern of expression, is the identity and source of the signal that leads to this regionalization. Several possibilities can be suggested for the formation of the *CdxA* stripe of expression. (a) The localization of the stripe is autonomous to the cells expressing the protein and independent of their surroundings. (b) The localization comes as a response to external signals such as a morphogen gradient. The first possibility would imply that the CdxA expressing cells are initially localized to the posterior marginal zone, and as a group they join the PS and move rostrally along it until they reach their final position at the full PS stage. Based on the available information on cell movements during PS elongation, it appears that this possibility is incorrect. Elongation of the PS has been studied in a number of instances (Bellairs, 1986; Malan, 1953; Nicolet, 1971; Rosenquist, 1966; Spratt, 1946; Stern, 1991; Schoenwolf, 1991) and from these experiments it has been concluded that the elongation comes about by a process similar to convergent extension (Bellairs, 1986; Stern, 1991). During this process, cells from the epiblast are recruited into the PS close to its anterior end and then they either move rostrally or caudally resulting in the overall increase in length of the streak. In other studies using carbon markings of the epiblast, it was concluded that the cells in the posterior half of the epiblast are hardly moving along the anterior-posterior axis (Spratt, 1946). In summary, these experiments suggest that along the rostrocaudal axis, cells in the posterior PS and the epiblast are either stationary or moving caudally, but no evidence was obtained for rostral movements.

The possibility of a morphogen gradient gets support from experiments in *Drosophila* embryos where this possibility has been proven (Driever et al., 1988; Hülskamp et al., 1990; Struhl et al., 1989; Hoch et al., 1991). Furthermore, the description of the patterns of expression of several genes during gastrulation has suggested that a network of regulatory proteins and growth factors is active at this developmental stage, and at least some of its members function or are affected by the regionalization of the anteroposterior axis, as evidenced from their patterns of expression (Blum et al., 1992; Dush and Martin, 1992; Niswander and Martin, 1992).

From early gastrulation up to the definitive streak stage, the stripe of *CdxA* expression exhibits an apparent anterior

'movement' as the PS elongates. This apparent anterior movement is probably not due to the anterior movement of the CdxA-positive cells as no such movement has been described (Bellairs, 1986; Malan, 1953; Nicolet, 1971; Rosenquist, 1966; Spratt, 1946; Stern, 1991). In the *Drosophila* embryo, a number of instances of gene regulation have been described where the transcriptional activity of one gene depends on the concentration of the protein product of another. Many of these cases involve DNA-binding proteins expressed as gradients in the fly embryo, and the genes that they regulate respond to different protein concentrations (Driever et al., 1988; Hülskamp et al., 1990; Struhl et al., 1989; Hoch et al., 1991). It has been shown that the response of the different genes is concentration dependent, and thresholds of protein amounts play a central role in establishing anterior and posterior boundaries of expression (Small et al., 1992; Ingham and Smith, 1992; St. Johnston and Nüsslein-Volhard, 1992). Thresholds have also been shown to play a role in vertebrate development (Green and Smith, 1990). Taking all this information together, it could be suggested that the *CdxA* stripe of expression is a response to morphogen gradient(s) along the axis of the primitive streak and a threshold model to establish the anterior and posterior boundaries of CdxA

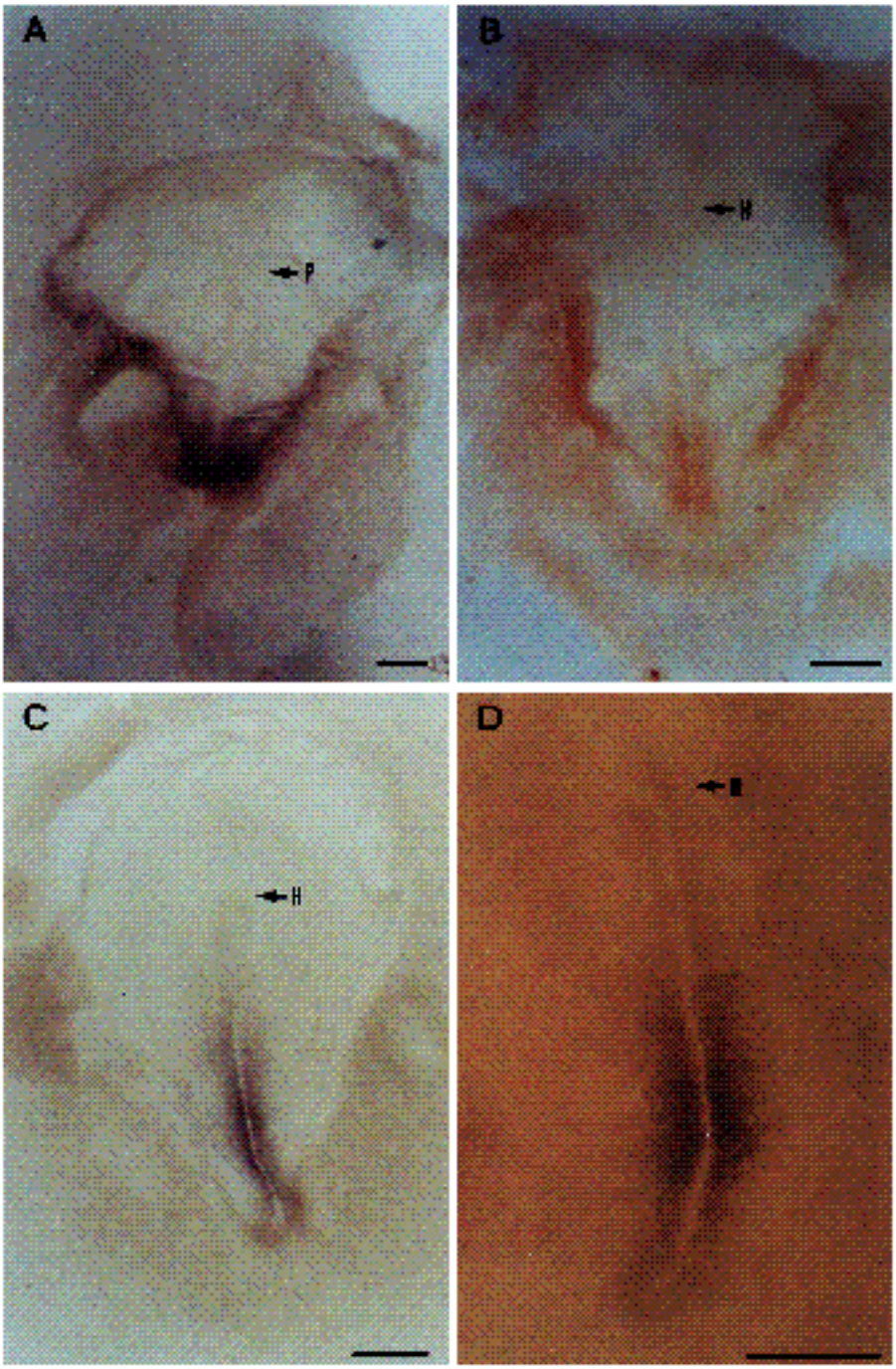

Fig. 2. Localization of the CdxA protein during primitive streak elongation. Whole-mount immunohistochemistry of chicken embryos at stages 3-3$^+$ (A), 3$^+$ (B), 4 (C) and 4$^+$ (D) with the 6A4αCdxA monoclonal antibody. The embryos are oriented rostral to the top. At the earliest stages detectable (A), the CdxA protein is found along most of the border of the area pellucida. At the posterior end of the embryo, cells localized further into the area pellucida and exhibiting higher staining levels can be detected. As migration from the epiblast and PS to form the mesoderm and endoderm begins, the staining previously localized at the posterior marginal zone appears to have moved and incorporated into the PS (B). As gastrulation continues, the staining along the border between the area pellucida and the area opaca disappears (C, D). Further, a portion of the primitive streak continues to express the CdxA protein and it expands rostrocaudally (C). As the primitive streak reaches its maximal length, the CdxA-expressing region exhibits a clear peak of expression about one third of the PS length from the caudal end. From the maximal region of expression, the CdxA level decreases in the anterior and posterior directions (D). Abbreviations P, primitive pit; H, Hensen's node. Scale bar equals 500 μm.

expression. The anterior 'movement' of the CdxA stripe of expression could be attributed to the possibility that the source of the factor that establishes the position of the *CdxA* stripe is also moving in the rostral direction. The primitive node or Hensen's node, which moves in a rostral direction as the PS elongates, fulfills the criteria for the source of such a factor. Recently, a number of genes have been described in *Xenopus*, whose expression localizes to Spemann's organizer. Some of these genes are putative regulatory genes like *goosecoid* (Blumberg et al., 1991), *fork*

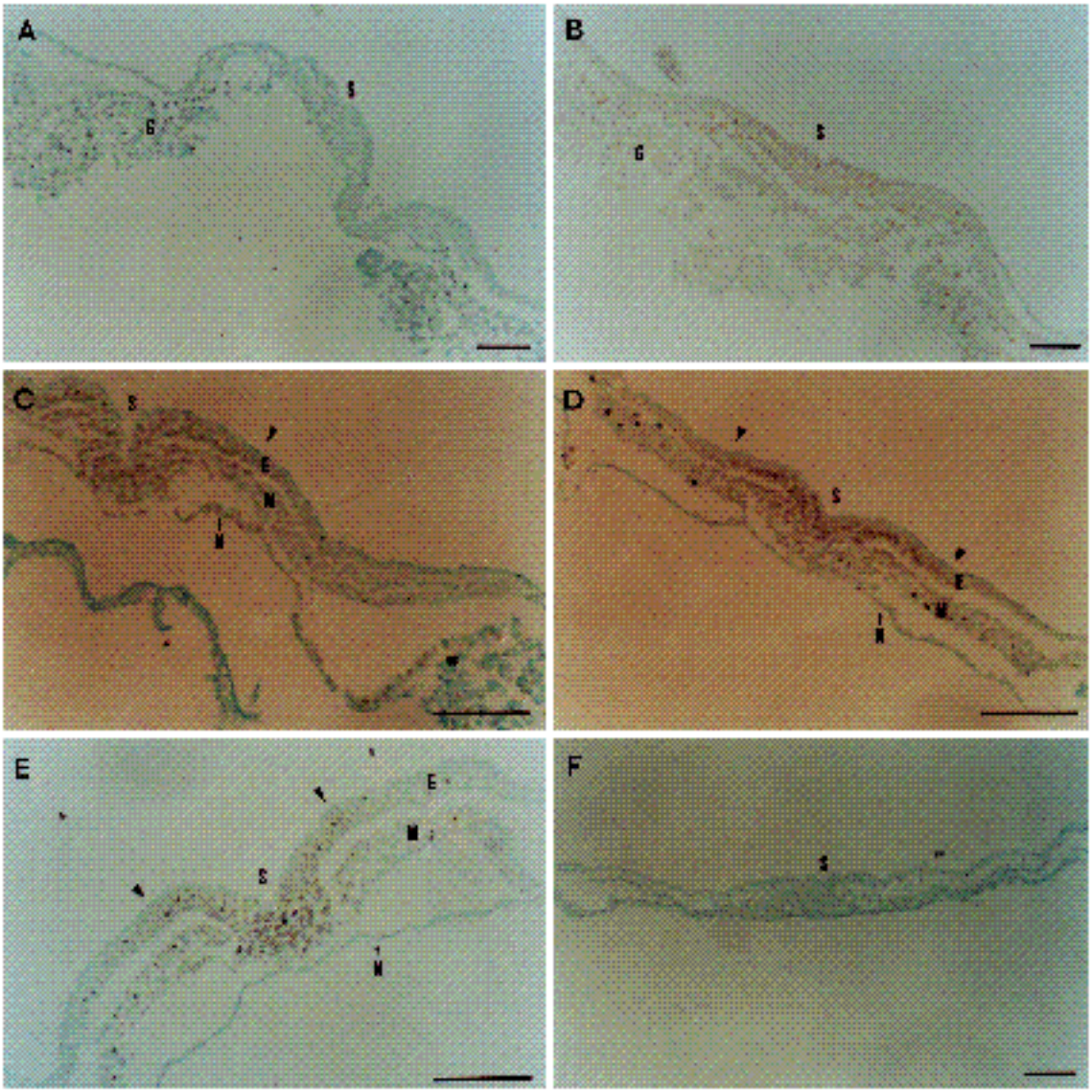

Fig. 3. Cell type localization of CdxA during gastrulation. Localization of the CdxA protein in tissue sections of embryos at different stages during elongation and regression of the PS. The sections correspond to embryos as in Figs 2 and 4. (A) Sections of embryos at stage 3 revealed that the staining at the boundary between the area pellucida and the area opaca is localized to the large yolky cells of the germ wall. (B) At stage 3^+, cross sections in the region of the stained PS showed the germ wall staining and in addition a lower level of staining can be observed in the primitive streak. (C) At the definitive streak stage (stage 4^+), on a cross section in the labeled region, the CdxA protein is present in all the cells of the PS. In the same embryos CdxA is expressed in the epiblast and newly formed mesoderm in the cells in the vicinity of the PS. In the epiblast expression is apparently in the cells moving towards the PS, while in the mesoderm, expression is in the cells leaving the PS. In both layers, there is a clear lateral boundary of expression (arrowheads). In the definitive endoderm, all the cells are labeled extending all the way to the marginal zone. (D,E) During PS regression, sections of embryos at stages 5 (D) and 8 (E) revealed a very similar situation as in embryos at definitive streak (C). While expression in the PS, epiblast and mesoderm is the same including the lateral boundary of expression (arrowheads), during regression no clear labeling of the definitive endoderm could be detected. The lack of endodermal labeling could be attributed to the cessation in the formation of endoderm through the streak. (F) At stage 10, to corroborate the lack of staining, embryos were serially sectioned confirming our observation by whole mounts. The embryo in A was stained in the presence of $NiCl_2$ resulting in a black precipitate, while the embryos (B-F) were stained in the absence of $NiCl_2$ resulting in a brown precipitate. Abbreviations E, epiblast; M, mesoderm; N, endoderm; S, primitive streak; G, germ wall. Scale bar equals 100 μm.

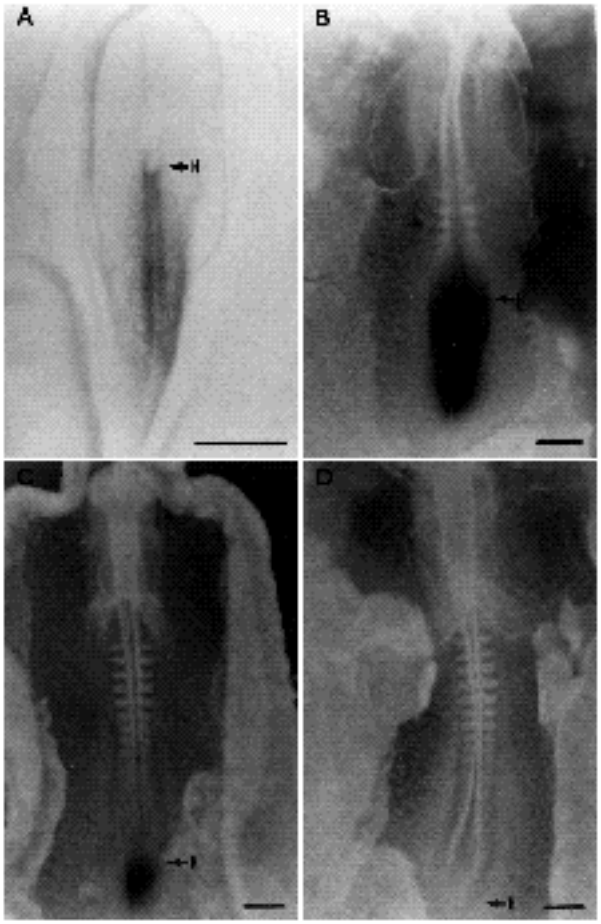

Fig. 4. CdxA expression during PS regression. Localization of the CdxA protein by whole-mount immunohistochemistry during regression of the PS, stages 5 (A), 8 (B), 9^+ (C) and 10^+ (D). (A) Soon after the onset of PS regression during head process stage distribution of the CdxA protein can be seen all along the PS. (B,C) As the PS continues its regression the CdxA protein remains restricted to the PS with Hensen's node as its anterior boundary. (D) At stage 10 (10 somites), the CdxA protein becomes undetectable and tail bud formation at the posterior end of the embryo begins(D). Abbreviations H, Hensen's node. Scale bar equals 500 μm.

head (Dirksen and Jamrich, 1992) and *Xlim-1* (Taira et al., 1992), or developmental factors like *noggin* (Smith and Harland, 1992). Comparisons of the functions of Spemann's organizer and Hensen's node and, more recently transplantations of Hensen's nodes to *Xenopus* embryos have suggested that these two structures might be at least partly homologous (Kintner and Dodd, 1991). These observations suggest that one of these genes, or an unknown one that behaves in a similar manner, could play a role in determining the position of the *CdxA* stripe and in the axial specification along the PS.

Formation of the definitive endoderm

Primitive streak elongation in the chicken embryo, from the onset of primitive streak formation to the appearance of the head fold primordium, takes about 12 hours, depending on the incubation conditions (Hamburger and Hamilton, 1951; Vakaet, 1970). During this time period, a number of different events take place such as: formation of the PS itself, recruitment of epiblast cells to the streak and migration, and formation of part of the mesodermal and the whole endodermal germ layers (Bellairs, 1986; Stern, 1991; Nicolet, 1971). The cell movements during the formation of the mesoderm and the endoderm have been described to a certain extent. The molecular events leading to the formation of the mesoderm are currently being deciphered (for reviews see Dawid, 1992; Green and Smith, 1991; Jessell and Melton, 1992; Niehrs and DeRobertis, 1992). Numerous factors that can influence mesoderm formation at different axial levels have been described, but the gene network responsible for the production of these factors is poorly understood. As regards the formation of the endodermal germ layer, our knowledge of the factors that lead to its formation, and the genes that regulate its morphogenesis, is even more fragmentary than in the mesodermal case. Little is known about genes expressed in the definitive endoderm during gastrulation, or about factors that can affect its formation (Minuth and Grunz, 1980) or about factors secreted by it (Kokan-Moore et al., 1991). The localization of the CdxA protein to the newly formed definitive endodermal layer raises the possibility that this gene plays part in the regulatory network responsible for the formation of this germ layer.

The definitive or gut endoderm arises from cells originally localized to the epiblast, which, after undergoing gastrulation through the PS together with the mesoderm, form the endodermal germ layer. A number of experimental observations have raised the question of the relationship between the mesodermal and the endodermal cell lineages during gastrulation. In a series of experiments using the HNK-1 antibody, it was possible to label, prior to gastrulation, cells destined to become mesoderm and endoderm (Stern and Canning, 1990). From these experiments, it was concluded that, prior to gastrulation and PS formation, the prospective mesoderm and endoderm cells share surface determinants and might even share a common ancestral cell type. It has previously been shown that removal of the endodermal layer during gastrulation results in the regeneration of the endodermal layer (England and Wakely, 1978;). From this regenerated layer, endodermal-derived tissues were observed. One of the interesting questions regarding this new layer is the origin of these cells. It was concluded that the observations pointed to mesodermal cells becoming endodermal (England and Wakely, 1978). This possibility has recently gained further support when transplantation of somites back into gastrulating embryos resulted in the generation of endodermal tissues from donor origin (Veini and Bellairs, 1991). During early gastrulation, the CdxA protein localizes to epiblast cells in the close vicinity of the PS and also to all cells in the streak. In addition, the newly formed mesodermal cells express this protein but, as they move away from the PS, they become negative. Moreover all definitive endodermal cells from the PS to the marginal zone express this protein as well. This pattern of expression also raises the possibility that in the region of the *CdxA* stripe, this gene is expressed in cells destined to migrate through the streak, and in all cells already migrating through the streak. The difference between the newly

formed mesoderm and the endoderm is that mesodermal cells become negative for the CdxA protein as they leave the PS while endodermal cells remain positive until stage 5 (H&H).

There is only one time window between stages 5 to 10 (H&H), during which the *CdxA* protein is undetectable in cells of endodermal origin (Frumkin et al., 1991; Frumkin and Fainsod, unpublished results). Interestingly, by the beginning of PS regression (stage 5), almost all the endodermal cells have migrated through the PS and from then on, mostly mesoderm is undergoing gastrulation (Nicolet, 1967). This observation supports the notion that *CdxA* plays a role in the formation of the endodermal germ layer. The early and late patterns of expression of *CdxA* suggest that this gene's function is related to some aspects in the establishment of the endodermal lineage and its derivatives such as the intestinal epithelium (Frumkin et al., 1991).

We wish to thank H. Eyal-Giladi for the stimulating discussions. Also we wish to thank G. Pillemer, H. Cedar, O. Khaner and Y. Yisraeli for critically reading the manuscript. This work was funded in part by a grant from The Council for Tobacco Research, USA to A. F.

REFERENCES

Azar, Y. and Eyal-Giladi, H. (1979). Marginal zone cells- the primitive streak inducing component of the primary hypoblast in the chick. *J. Embryol. Exp. Morph.* **52**, 79-88.

Bellairs, R. (1986). The primitive streak. *Anat. Embryol.* **174**, 1-14.

Blum, M., Gaunt, S. J., Cho, K. W. Y., Steinbeisser, H., Blumberg, B., Bittner, D. and De Robertis, E. M. (1992). Gastrulation in the mouse: The role of homeobox gene goosecoid. *Cell* **69**, 1097-110.

Blumberg, B., Wright, C. V. E., De Robertis, E. M. and Cho, K. W. Y. (1991). Organizer-specific homeobox genes in *Xenopus laevis* embryos. *Science* **253**, 194-196.

Brosius, J. (1984). Toxicity of an overproduced foreign gene product in *Eschericia Coli* and its use in plasmid vectors for the selection of transcription terminators. *Gene* **27**, 161-172.

Cho, K. W. Y., Blumberg, B., Steinbeisser, H. and De Robertis, E. (1991). Molecular nature of Spemann's organizer: the role of the Xenopus homeobox gene *goosecoid*. *Cell* **67,** 1111-1120.

Davis, C. A., Holmyrad, D. P., Millen, K. J., and Joyner, A. L. (1991). Examining pattern formation in mouse, chicken and frog embryos with an En-specific antiserum. *Development* **111**, 287-298.

Dawid, I. B. (1992). Mesoderm induction and axis determination in *Xenopus laevis. BioEssays* **14,** 687-691.

Dent, J. A., Polson, A. G., and Klymkowsky, M. W. (1989). A whole mount immunocytochemical analysis of the expression of the intermediate filament protein vimentin in Xenopus. *Development* **105**, 61-74.

Dirksen, M. L. and Jamrich, M. (1992). A novel, activin-inducible, blastopore lip-specific gene of *Xenopus laevis* contains a *fork head* DNA-binding domain. *Genes Dev.* **6,** 599-608.

Driever, W. and Nüsslein-Volhard, C. (1988). The *bicoid* protein determines position in the Drosophila embryo in a concentration-dependent manner. *Cell* **54,** 95-104.

Dush, M. K. and Martin, G. R. (1992). Analysis of mouse Evx genes: Evx-1 displays graded expression in the primitive streak. *Dev. Biol.* **151**, 273-287.

England, M. A. and Wakely, J. (1978). Endoderm regeneration in the chick embryo studied by SEM. *Anat. Embryol.* **154**, 55-66.

Frumkin, A., Rangini, Z., Ben-Yehuda, A., Gruenbaum, Y. and Fainsod, A. (1991). A chicken caudal homologue, Chox-cad, is expressed in the epiblast with posterior localization and in the early endodermal lineage. *Development* **112**, 207-219.

Gaunt, S. J. (1987). Homoeobox gene *Hox-1.5* expression in mouse embryos: earliest detection by *in situ* hybridization is during gastrulation. *Development* **101**, 51-60.

Gerhart, J., Danilchik, M., Doniach, T., Roberts, S., Rowning, B. and Stewart, R. (1989). Cortical rotation in the Xenopus egg: consequences for the anteroposterior pattern of embryonic dorsal development. *Development* **Supplement 107,** 37-52.

Green, J. B. A. and Smith, J. C. (1990). Graded changes in dose of a *Xenopus* activin A homologue elicit stepwise transitions in embryonic cell fate. *Nature* **347,** 391-394.

Green, J. B. A. and Smith, J. C. (1991). Growth factors as morphogens: do gradients and thresholds establish body plan? *Trends Genet.* **7,** 245-250.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88**, 49-92.

Harlow, E. and Lane, D. (1988). *Antibodies A Laboratory Manual*. Cold Spring Harbor Laboratory, New York.

Hoch, M., Seifert, E. and Jäckle, H. (1991). Gene expression mediated by *cis*-acting sequences of the Krüppel gene in response to the *Drosophila* morphogens *bicoid* and *hunchback. EMBO J.* **10**, 2267-2278.

Hülskamp, M. and Tautz, D. (1991). Gap genes and gradients-the logic behind the gaps. *BioEssays* **13**, 261-268.

Hülskamp, M., Pfeifle, C. and Tautz, D. (1990). A morphogenetic gradient of *hunchback* protein organizes the expression of the gap genes *Krüppel* and *knirps* in the early *Drosophila* embryo. *Nature* **346,** 577-580.

Ingham, P. and Smith, J. (1992). Crossing the threshold. *Current Biol.* **2,** 465-467.

Jessell, T. M. and Melton, D. A. (1992). Diffusible factors in vertebrate embryonic induction. *Cell* **68**, 257-270.

Keller, R., Shih, J. and Wilson, P. (1991). Cell motility, control and function of convergence and extension during gastrulation in *Xenopus*. In *Gastrulation: Movements, Patterns and Molecules* (ed. R. Keller, W. H. Clark, Jr., F. Griffin), p. 101-120. New York: Plenum Press.

Khaner, O. and Eyal-Giladi, H. (1986). The embryo-forming potency of the posterior marginal zone in stages X through XII of the chick. *Dev. Biol.* **115**, 275-281.

Kintner, C. R. and Dodd, J. (1991). Hensen's node induces neural tissue in *Xenopus* ectoderm. Implications for the action of the organizer in neural induction. *Development* **113,** 1495-1505.

Kokan-Moore, N. P., Bolender, D. L. and Lough, J. (1991). Secretion of inhibin ßa by endoderm cultured from early embryonic chicken. *Dev. Biol.* **146,** 242-245.

Malan, M. E. (1953). The elongation of the primitive streak and the localization of the presumtive chorda-mesoderm in the early chick blastoderm studied by means of coloured marks with nile blue sulphate. *Arch. Biol.* **64,** 149-188.

Minuth, M. and Grunz, H. (1980). The formation of mesodermal derivatives after induction with vegetalizing factors depends on secondary cell interactions. *Cell Diff.* **9,** 229-238.

Mitrani, E., Ziv, T., Thomsen, G., Shimoni, Y., Melton, D. A. and Bril, A. (1990). Activin can induce the formation of axial structures and is expressed in the hypoblast of the chick. *Cell* **63,** 495-501.

Nicolet, G. (1967). La chronologie d'invagination chez le Poulet: Etude a l'aide de la thymidine tritiee. *Experientia* **23**, 576-577.

Nicolet, G. (1970). Analyse autoradiographique de la localisationdes diverses ébauches présomptives dans la ligne primitive de l'embryon de poulet. *J. Embryol. Exp. Morph.* **23**, 79-108.

Nicolet, G. (1971). Avian gastrulation. *Adv. Morphog.* **9**, 231-262.

Niehrs, C. and De Robertis, E. M. (1992). Vertebrate axis formation. *Curr. Opinion Genet. Dev.* **2,** 550-555.

Niswander, L. and Martin, G. R. (1992). *Fgf-4* expression during gastrulation, myogenesis, limb and tooth development in the mouse. *Development* **114,** 755-768.

Nusse, R. and Varmus, H. E. (1992). *Wnt* genes. *Cell* **69,** 1073-1087.

Rosa, F. M. (1989). *Mix.1*, a homeobox mRNA inducible by mesoderm inducers, is expressed mostly in the presumptive endodermal cells of Xenopus embryos. *Cell* **57,** 965-974.

Rosenquist, G. C. (1966). A radioautographic study of labeled grafts in the chick blastoderm. Development from primitive streak stages to stage 12. *Contrib. Embryol. Carnegie Inst. Wash.* **38**, 111-121.

Schoenwolf, G. C. (1979). Histological and ultrastructural obsevations of tail bud formation in the chick embryo. *Anat Rec* **193**, 131-148.

Schoenwolf, G. C. (1991). Cell movements in the epiblast during gastrulation and neurulation in avian embryos. In *Gastrulation: Movements, Patterns and Molecules* (ed. R. Keller, W. H. Clark, Jr., F. Griffin), p. 1-27. New York: Plenum Press.

Slack, J. M. W. and Tannahill, D. (1992). Mechanisms of anteroposterior axis specification in vertebrates. Lessons from the amphibians. *Development* **114,** 285-302.

Small, S., Blair, A. and Levine, M. (1992). Regulation of *even-skipped* stripe 2 in the *Drosophila* embryo. *EMBO J.* **11,** 4047-4057.

Smith, D. B. and Johnson, K. S. (1988). Single-step purification of polypeptides expressed in Escherichia Coli as fusions with glutathione S-transferase. *Gene* **67,** 31-40.

Smith, W. C. and Harland, R. M. (1992). Expression cloning of noggin, a new dorsalizingfactor localized to the Spemann organizer in Xenopus embryos. *Cell* **70,** 829-840.

Spratt, N. T. Jr. (1946). Formation of the primitive streak in the explanted chick blastoderm marked with carbon particles. *J. Exp. Zool.* **103**, 259-304.

St Johnston, D. and Nusslein-Volhard, C. (1992). The origin of pattern and polarity in the Drosophila embryo. *Cell* **68**, 201-209.

Stern, C. D. (1990). The marginal zone and its contribution to the hypoblast and primitive streak of the chicke embryo. *Development* **109,** 667-682.

Stern, C. D. and Canning, D. R. (1990). Origin of cells giving rise to mesoderm and endoderm in chick embryo. *Nature* **343,** 273-275.

Stern, C. D. (1991). Mesoderm formation in the chick embryo, revisited. In *Gastrulation: Movements, Patterns and Molecules* (ed. R. Keller, W. H. Clark, Jr., F. Griffin), p. 29-41. New York: Plenum Press.

Struhl, G., Struhl, K. and Macdonald, P. M. (1989). The gradient morphogen bicoid is a concentration-dependent transcriptional activator. *Cell* **57**, 1259-1273.

Sundin, O. H., Busse, H. G., Rogers, M. B., Gudas L. J. and Eichele G. (1990). Region-specific expression in early chick and mouse embryos of Ghox-lab and Hox 1.6, vertebrate homeobox-containing genes related to *Drosophila labial. Development* **108**, 47-58.

Taira, M., Jemrich, M., Good, P. J. and Dawid, I. B. (1992). The LIM domain-containing homeo box gene *Xlim-1* is expressed specifically in the organizer region of *Xenopus* gastrula embryos. *Genes Dev.* **6**, 356-366.

Thomsen, G., Woolf, T., Whitman, M., Sokol, S., Vaughan, J., Vale, W. and Melton, D. A. (1990). Activins are expressed early in Xenopus embryogenesis and can induce axial mesoderm and anterior structures. *Cell* **63,** 485-493.

Vakaet, L. (1970). Cinephotomicrographic investigations of gastrulation in the chick blastoderm. *Arch. Biol.* **81**, 387-426.

Veini, M. and Bellairs, R. (1991). Early mesoderm differentiation in the chick embryo. *Anat. Embryol.* **183,** 143-149.

Ziv, T., Shimoni, Y. and Mitrani, E. (1992). Activin can generate ectopic axial structures in chick blastoderm explants. *Development* **115,** 689-694.

(Accepted 8 March 1993)